

05-22-02

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TRANSMITTAL LETTER
(General - Patent Pending)

Docket No.
ISPH-0463
In Re Application Of: **Monia et al.**

Serial No

09/575,554

Filing Date

May 22, 2000

Examiner

Jeffrey N. Fredman

Group Art Unit

1637

Title: **ANTISENSE OLIGONUCLEOTIDE INHIBITION OF RAS**
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Dated: **May 20, 2002**

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ISPH-0463

Inventors:

Monia et al.

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J. Fredman

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Antisense Oligonucleotide Inhibition of
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Dear Sir:

APPEAL BRIEF

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EXHIBIT I	Pending Claims
EXHIBIT II	U.S. Patent 6,117,848
EXHIBIT III	Notice of Allowance for U.S. Serial No. 09/128,494 Issued as U.S. Patent 6,117,848

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I. Real Party of Interest

The real party of interest is Isis Pharmaceuticals, Inc., assignee of all rights, title and interest in the instant application.

II. Related Appeals and Interferences

There are no related appeals or interferences.

III. Status of Claims

This application is a continuation-in-part of U.S. Patent Application Serial No. 09/128,494, filed August 3, 1998, now U.S. Patent 6,117,848, which is a continuation-in-part of U.S. Application Serial No. 08/411,734, filed April 3, 1995, which is a continuation of PCT/US93/09346, filed October 1, 1993, which is a continuation-in-part of U.S. Patent Application Serial No. 07/958,134, filed October 5, 1992, abandoned, and U.S. Patent Application Serial No. 08/007,996, filed January 21, 1993, abandoned, all of which are assigned to the assignee of the present invention.

An Office Action was mailed on March 22, 2001 wherein claims 1 through 23 were rejected. Specifically, claim 6 was rejected under 35 U.S.C. 101, as claiming the same invention as that of claim 1 of U.S. Patent No. 5,872,242. Claims 1, 4, 5 and 7-22 were rejected under the judicially created doctrine of obviousness-type

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double patenting as being unpatentable over claims 1-8 of U.S. Patent No. 5,872,242. Claims 1, 2, 5 and 7-23 were rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 1-4 of U.S. Patent No. 5,576,208. Claims 1, 2, 5 and 7-23 also were rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 1-6 of U.S. Patent No. 5,582,986. Claims 1-5 and 7-23 were rejected under 35 U.S.C. 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor, at the time the application was filed, had possession of the claimed invention. Claims 1, 3 and 5-22 were rejected under 35 U.S.C. § 103(a) as being unpatentable over Bos (U.S. Patent No. 4,871,838), Daaka et al., Hall et al. (1985) and Saison-Behmoaras et al. (1991), each in view of Uhlmann et al., Agrawal et al., Inoue et al., and further in view of Smith.

A response was filed May 21, 2001 wherein claims 2-6 and 21-23 were canceled and claims 1, 11, 13 and 14 were amended.

In an Office Action dated November 15, 2001, pending claims 1 and 7-20 were finally rejected. Specifically, claim 6 was rejected under 35 U.S.C. 101, as claiming the same invention as that of claim 1 of U.S. Patent No. 5,872,242. Claims 1, 2, 5 and 7-23 were rejected under the judicially created doctrine of obviousness-type

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double patenting as being unpatentable over claims 1-4 of U.S. Patent No. 5,576,208. Claims 1, 2, 5 and 7-23 also were rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 1-6 of U.S. Patent No. 5,582,986. Claims 1 and 7-20 were rejected under 35 U.S.C. § 103(a) as being unpatentable over Bos (U.S. Patent No. 4,871,838), Daaka et al., Hall et al. (1985) and Saison-Behmoaras et al. (1991), each in view of Uhlmann et al., Agrawal et al., Inoue et al., and further in view of Smith.

A response was filed by Appellant on February 11, 2002.

In an Advisory Action dated March 5, 2002, the final rejection of claims 1 and 7-20 was maintained.

Accordingly, the rejection of claims 1 and 7-20 under 35 U.S.C. § 103(a) is on appeal. A copy of pending claims 1 and 7-20 is attached hereto as Exhibit 1.

IV. Status of Amendments

All amendments to the claims have been entered upon this appeal.

V. Summary of the Invention

The claimed invention includes compositions and methods for inhibition of the expression of Ki-ras, where the compositions are antisense oligonucleotides targeted to specific regions of Ki-ras

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and are claimed according to specific sequences. The methods as claimed are methods for modulating expression of human Ki-ras and methods of preventing or treating conditions arising from the activation of a Ki-ras oncogene, all of which are based on administration of the antisense oligonucleotides of the present invention.

The claimed compositions and methods of use of the composition both are clearly described in the specification as filed, in particular at pages 35 through 65.

VI. Issues

The issue on appeal is whether claims 1 and 7-20 are unpatentable under 35 U.S.C. § 103(a) as being obvious over Bos (U.S. Patent No. 4,871,838), Daaka et al., Hall et al. (1985) and Saison-Behmoaras et al. (1991), each in view of Uhlmann et al., Agrawal et al., Inoue et al., and further in view of Smith.

VII. Grouping of Claims

Claims 1 and 7-20 stand or fall together on the issue of obviousness under 35 U.S.C. § 103(a) over Bos (U.S. Patent No. 4,871,838), Daaka et al., Hall et al. (1985) and Saison-Behmoaras et al. (1991), each in view of Uhlmann et al., Agrawal et al., Inoue et al., and further in view of Smith.

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VIII. Arguments

Issue: Whether claims 1 and 7-20 are obvious in light of Bos (U.S. Patent No. 4,871,838), Daaka et al., Hall et al. (1985) and Saison-Behmoaras et al. (1991), each in view of Uhlmann et al., Agrawal et al., Inoue et al., and further in view of Smith.

1. Examiner's basis for rejection

The Examiner has rejected claims 1 and 7-20 under 35 U.S.C. § 103(a) as being unpatentable over Bos (U.S. Patent No. 4,871,838), Daaka et al., Hall et al. (1985) and Saison-Behmoaras et al. (1991), each in view of Uhlmann et al., Agrawal et al., Inoue et al., and further in view of Smith. The Examiner suggests that the cited references teach and suggest every element and provide motivation to make the compounds. Specifically, the Examiner suggests that Bos provides the requisite sequence information for Ki-ras, that Daaka et al. disclose antisense oligonucleotides to the translation initiation site of human H-ras and their use to inhibit expression of H-ras, and that Saison-Behmoaras et al. teach oligonucleotides that specifically hybridize to the codon 12 region of H-ras and methods to inhibit gene expression. The teaching of these primary references is suggested by the Examiner to be supplemented by the teaching of modification to antisense oligonucleotides by Uhlmann et al., Agrawal et al., and Inoue et al., while Smith et al. teach use of antisense compounds against

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oncogenes or genes that are differentially expressed in tumor cells as treatment for cancer. The Examiner goes on to suggest that motivation to make antisense to Ki-ras is provided by Saison-Behmoaras et al., Daaka et al., and Bos et al. in their teaching of antisense to ras in general and the fact that Ki-ras is structurally and functionally equivalent to H-ras and N-ras. The Examiner then suggests that the reasonable expectation of success is provided by the teaching of Daaka et al. and Saison-Behmoaras et al. relating to antisense to H-ras.

2. Summary of cited prior art teachings

Bos et al. (US Patent 4,871,838) teach probes for use in detecting mutations in ras oncogenes, including the activity of specific complementary (antisense) oligonucleotides to human H-ras, but only the general activity of complementary oligonucleotides to Ki-ras that have from 12 to 43 nucleotides. Nowhere does this patent teach or suggest the specific sequences of the instant invention comprising from 8 to 30 nucleobases to which the claims as amended were limited and are now on appeal. Further, this patent fails to provide data showing specific activity of antisense oligonucleotides to Ki-ras to inhibit expression of Ki-ras DNA or RNA molecules.

Hall and Brown (1985) teach the sequence of human N-ras and mutations to codons 12 and 61. Nowhere does this paper teach or

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suggest antisense to Ki-ras, or any of the specific antisense oligonucleotide sequences of the instant invention.

Daaka et al. teach antisense oligonucleotides to the translation initiation codon of H-ras. However, nowhere does this prior art reference teach or suggest antisense compounds to Ki-ras, or any of the antisense oligonucleotide sequences of the instant invention.

Saison-Behmoaras et al. (1991) teach short modified antisense oligonucleotides targeted to H-ras and the 12th codon region as a target within the H-ras gene. Again, however, nowhere does this paper teach or suggest antisense compounds to Ki-ras, or any of the antisense oligonucleotide sequences of the instant invention.

Therefore, these primary references fail to teach or suggest the claims as amended which are limited to specific sequences of antisense compounds targeted to Ki-ras. The secondary references cited by the Examiner fail to overcome the deficiencies of the primary prior art references.

Uhlmann et al. discloses modifications to antisense oligonucleotides such as are taught in the instant invention. However, nowhere does this paper teach or suggest antisense oligonucleotides to human ras genes, nor any sequences taught by the instant invention.

Inoue et al. and Agrawal et al. teach ways to confer sensitivity and resistance to RNase H in cells. Nowhere do these

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papers teach or suggest antisense oligonucleotides to human ras genes, nor any sequences taught by the instant invention.

Finally, Smith et al. disclose antisense oligonucleotides as a tool for treatment of cancer. However, nowhere does this paper teach or suggest antisense oligonucleotides to human ras genes, nor any sequences taught by the instant invention.

3. The combination of cited art fails to establish a *prima facie* case of obviousness.

Applicants respectfully point out that the claims were amended during prosecution to be limited to specific SEQ ID NO's for antisense compounds targeted to specific regions within the sequence of Ki-ras. None of the primary references, either alone or combined, cited by the Examiner teach antisense compounds with the specific sequences as claimed that are targeted to Ki-ras. Although Bos et al. teach the sequence of Ki-ras itself, and the general idea of using oligonucleotides complementary to Ki-ras as probes for mutated Ki-ras forms, this reference does not teach successful use of Ki-ras antisense oligonucleotides to inhibit Ki-ras expression as claimed, nor does it teach the specific antisense oligonucleotide sequences as claimed which are targeted to Ki-ras. None of the other references cited teach antisense compounds of any type or sequence targeted to Ki-ras. A mere teaching of antisense to another target, or even to the same

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general target, is not sufficient to enable specific antisense sequences as claimed.

To establish a *prima facie* case of obviousness, three basic criteria must be met. MPEP 2143. First, there must be some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings. Second, there must be a reasonable expectation of success. Finally, the prior art must teach or suggest all claim limitations.

The cited combination of art fails to meet all these criteria.

Specifically, the combination of prior art cited fails to teach or suggest all of the limitations of the claims, which claim specific antisense oligonucleotide sequences targeted to human Ki-ras. The references also fail to give an expectation of success with the instant invention based on the fact that none of them teach successful inhibition of Ki-ras expression using antisense. A mere teaching of antisense to another target does not provide one of skill with the information necessary to predict that antisense to an entirely different target will be successful at inhibiting gene expression. It is only with the specification in hand that one of skill is able to see that certain antisense compounds were useful both *in vitro* and *in vivo* to inhibit Ki-ras expression. Accordingly, the references fail to establish a case of *prima facie* obviousness.

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In fact, in the patent application Serial No. 09/128,494 which issued as U.S. Patent No. 6,117,848, this Examiner (Examiner Fredman) found that a claim to "an oligonucleotide 8 to 30 nucleotides in length which is targeted to a nucleic acid encoding human Ki-ras and which is capable of inhibiting Ki-ras expression, wherein said oligonucleotide comprises SEQ ID NO: 20, 21, 22, 26, 28, 31 or 32" was non-obvious over the same combination of cited art (U.S. Patent 6,117,848 is attached hereto as Exhibit 2; see claim 9). Further, in his "Reasons for Allowance" (attached hereto as Exhibit 3), the Examiner states that Applicant had demonstrated for these specific sequences the secondary consideration of unexpected results. When Applicants' attorney discussed the pending claims and issued claim 9 of the 6,117,848 patent with the Examiner, it appears that the Examiner is making a distinction between "an oligonucleotide 8 to 30 nucleobases which comprises" a specific SEQ ID NO. and "an oligonucleotide 8 to 30 nucleobases which comprises at least an 8-nucleobase portion" of a particular SEQ ID NO. However, neither of these claims, either pending or issued, is directed to a specific oligonucleotide only as the Examiner seems to be suggesting. Both claims use "comprising" not "consisting of" language. In both cases the oligonucleotide is 8 to 30 mer and comprises some portion of a particular SEQ ID NO. Most importantly, no compounds having structural similarity to the antisense compounds as claimed, which is the Examiner's stated

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grounds of distinction, are taught or suggested by the cited combination of art.

Therefore, solely on the basis of the requirements for establishing a *prima facie* case of obviousness (MPEP 2143), these combined references fail to make claims 1 and 7-20 obvious.

4. Unpredictability of the art of antisense compounds must be considered

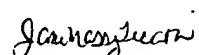
A *prima facie* case of obviousness based on structural similarity is rebuttable by proof that the claimed compounds possess unexpected advantages or superior properties (MPEP 2144.09; *In re Papesch*, 315 F.2d 381, 137 USPQ 43, CCPA 1963). Data provided in the specification as filed at pages 21-22, Table 2, and page 41, Table 11, clearly show that antisense oligonucleotides targeted to ras in general function to reduce ras expression in some cases, while others selected from the same regions failed to achieve the same results. In contrast, the compounds as claimed, which are limited to specific sequences, were shown at pages 35-38 and Figure 6 of the specification to have specific activity. Therefore, the fact that only certain antisense compounds targeted to ras in general may have the ability to inhibit gene expression reveals to one skilled in the art that success would not be expected based on the use of references showing activity of antisense to a target other than Ki-ras.

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IX. Conclusion

The references cited by the Examiner clearly do not provide the requisite teaching or suggestion to render the claimed invention obvious.

Respectfully submitted,



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EXHIBIT I

1. An oligonucleotide 8 to 30 nucleotides in length which is targeted to a nucleic acid encoding human Ki-ras, wherein said oligonucleotide is capable of inhibiting Ki-ras expression, and wherein said oligonucleotide comprises at least an 8-nucleobase portion of SEQ ID NO: 20, 21, 22, 26, 28, 31, 32 or 33.

7. The oligonucleotide of claim 1 which comprises at least one backbone modification.

8. The oligonucleotide of claim 1 wherein at least one of the nucleotide units of the oligonucleotide is modified at the 2' position of the sugar.

9. The oligonucleotide of claim 1 which is a chimeric oligonucleotide.

10. The oligonucleotide of claim 1 in a pharmaceutically acceptable carrier.

11. A method of modulating the expression of human Ki-ras comprising contacting tissues or cells containing a human Ki-ras gene with an effective amount of an oligonucleotide of claim 1 whereby expression of Ki-ras is modulated.

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12. A method of inhibiting the proliferation of cancer cells comprising contacting cancer cells with an effective amount of an oligonucleotide of claim 1, whereby proliferation of the cancer cells is inhibited.

13. A method of preventing or treating a condition arising from the activation of a Ki-ras oncogene comprising contacting an animal suspected of having a condition arising from the activation of a Ki-ras oncogene with an effective amount of an oligonucleotide of claim 1, whereby said condition is prevented or treated.

14. The method of claim 13 wherein said activation of a Ki-ras oncogene is abnormal expression of a Ki-ras oncogene.

15. The method of claim 13 wherein said condition is a hyperproliferative condition.

16. The method of claim 13 wherein the condition is cancer.

17. The method of claim 13 wherein the condition is colorectal cancer, melanoma, liposarcoma, mesothelioma, sarcoma, colon cancer, or pancreatic cancer.

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18. The method of claim 11 wherein the cells are cancer cells.

19. The method of claim 11 wherein the cells are blood cells.

20. The method of claim 11 wherein the cells are peripheral blood mononuclear cells.

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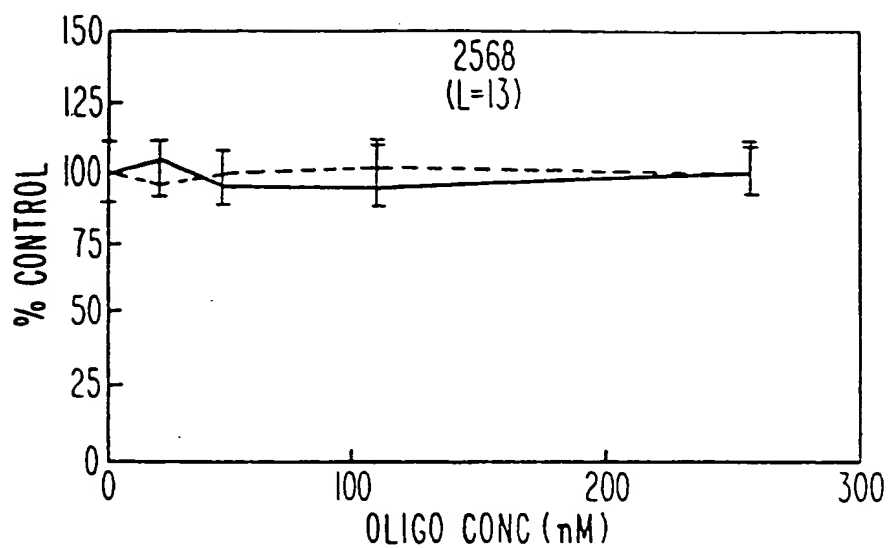
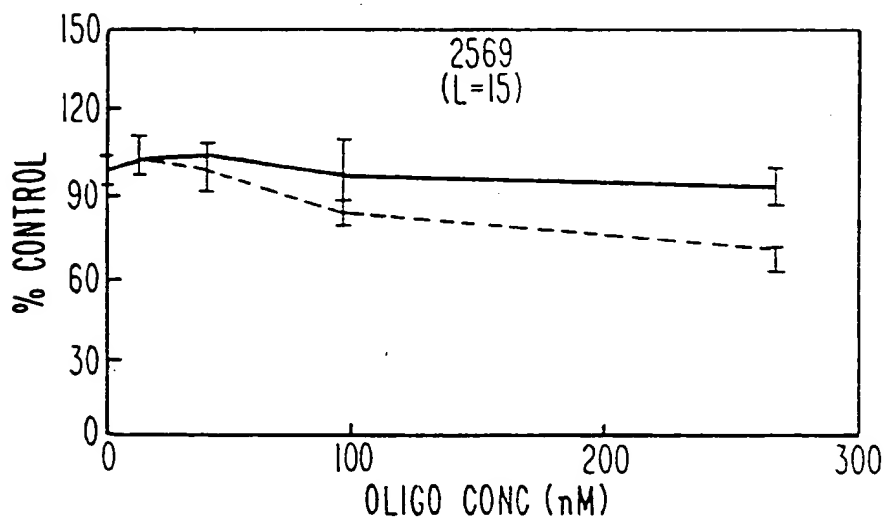
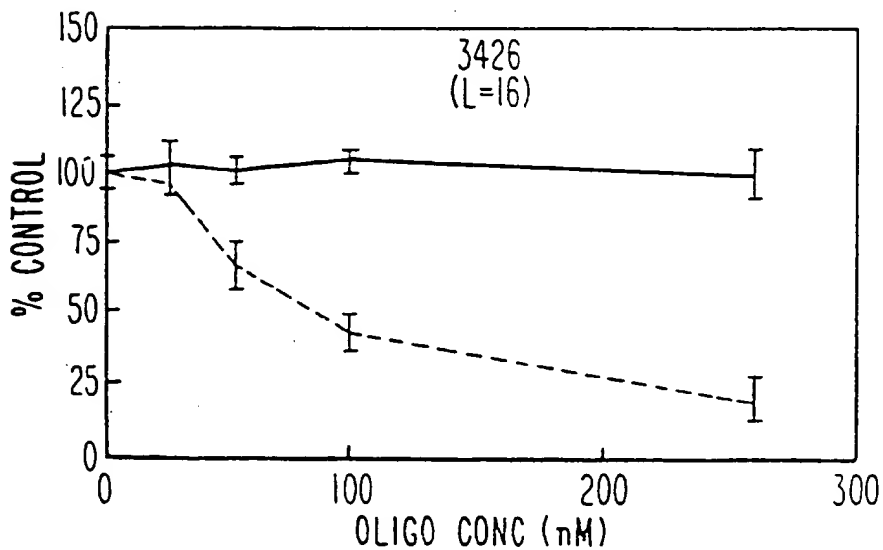
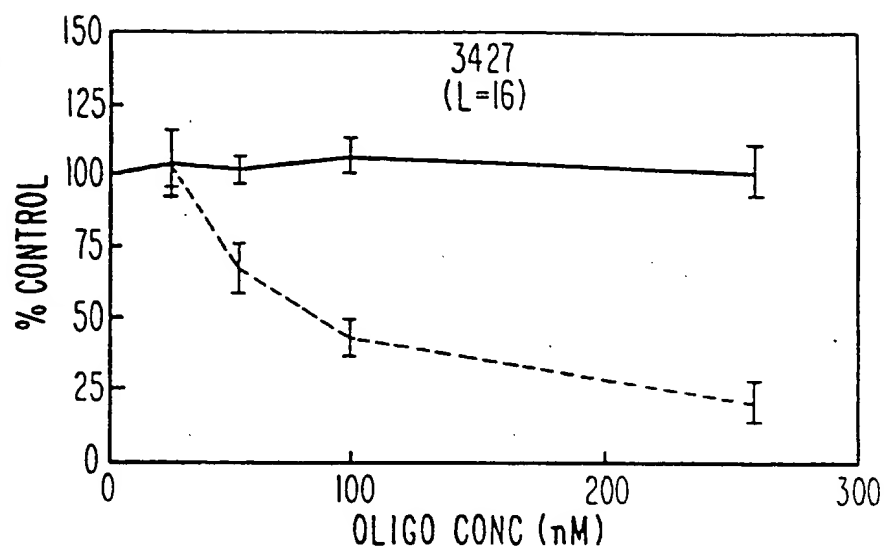
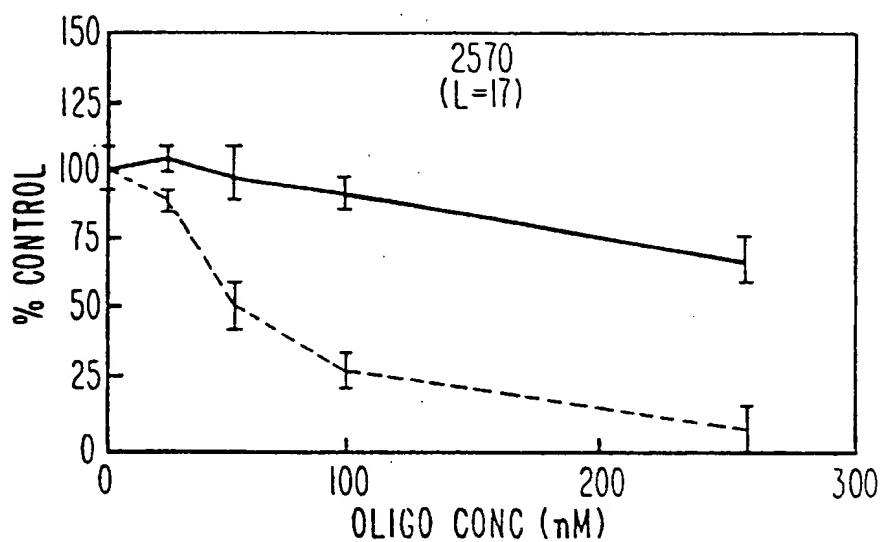
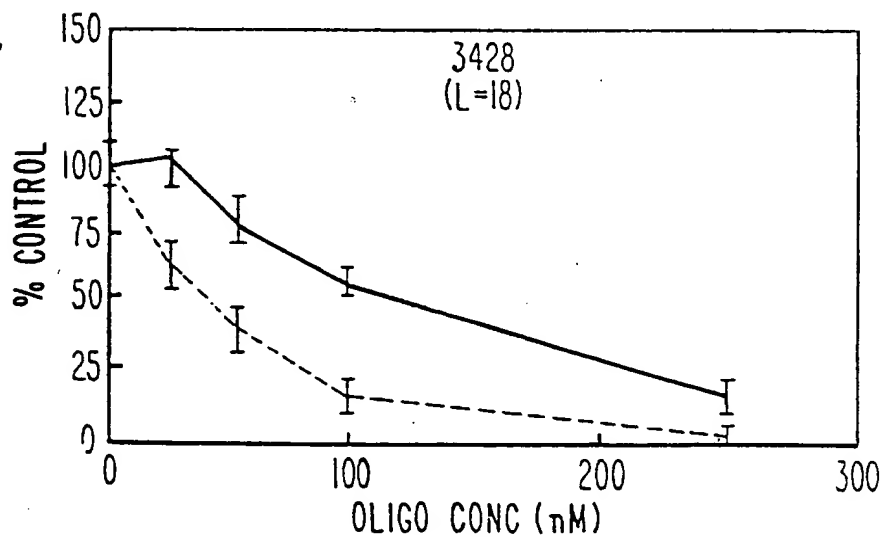
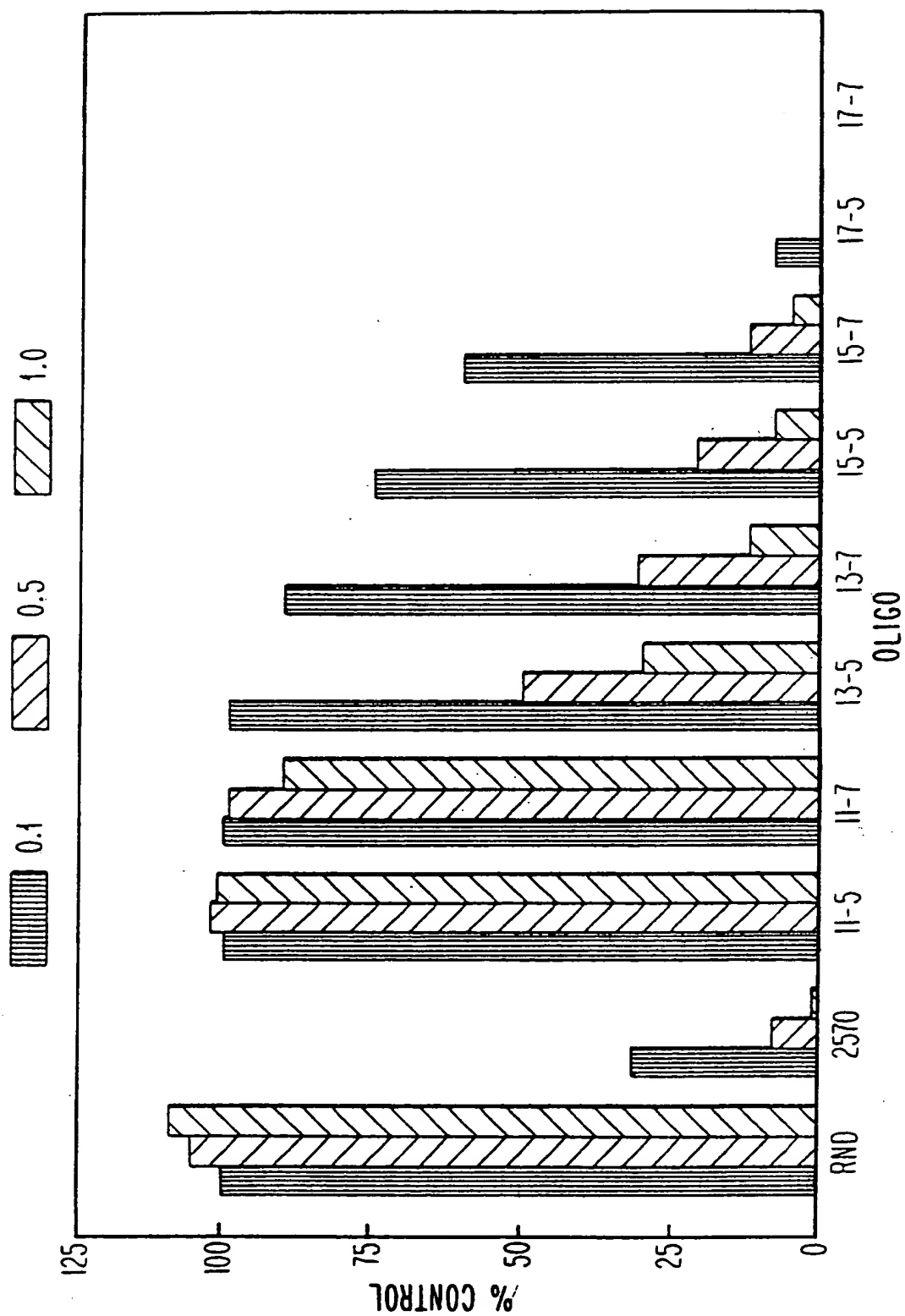
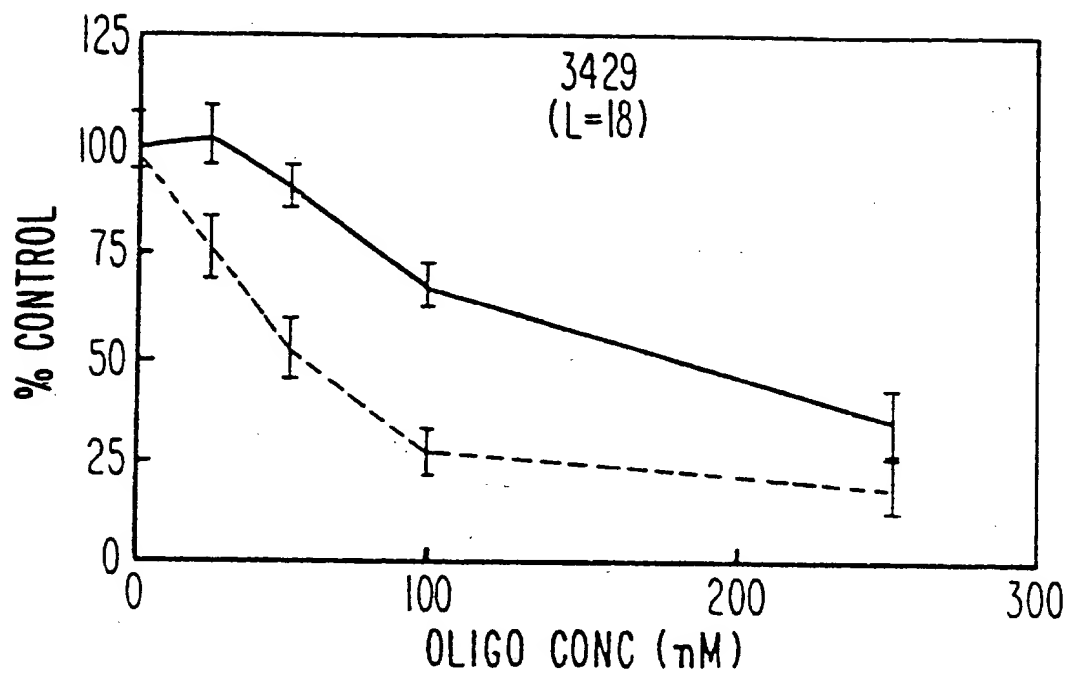
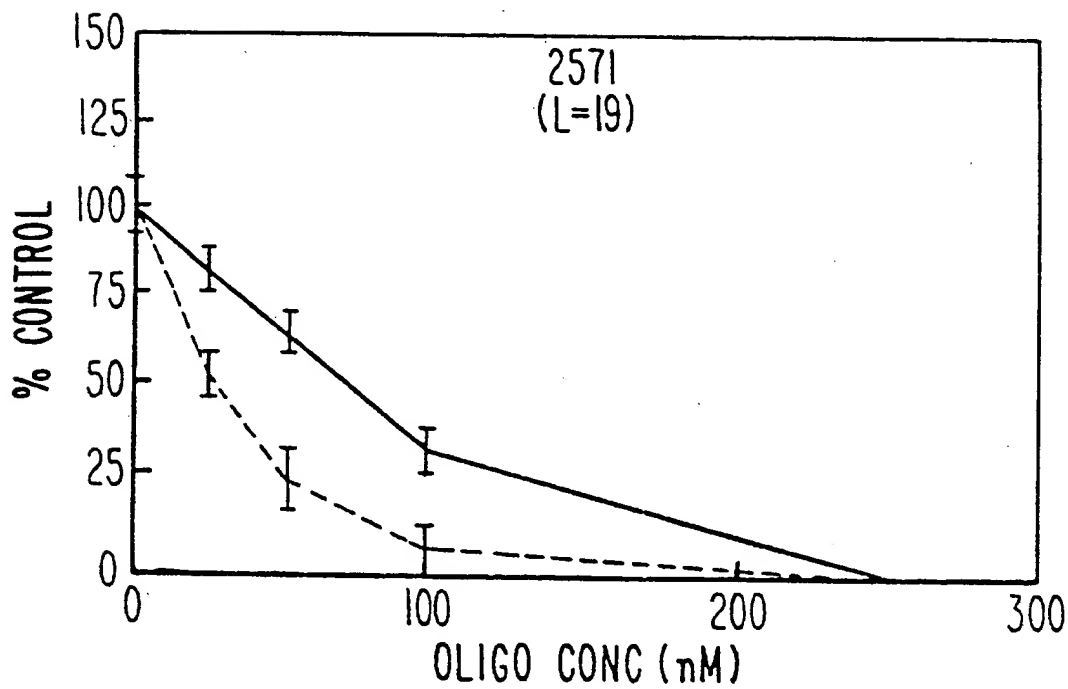
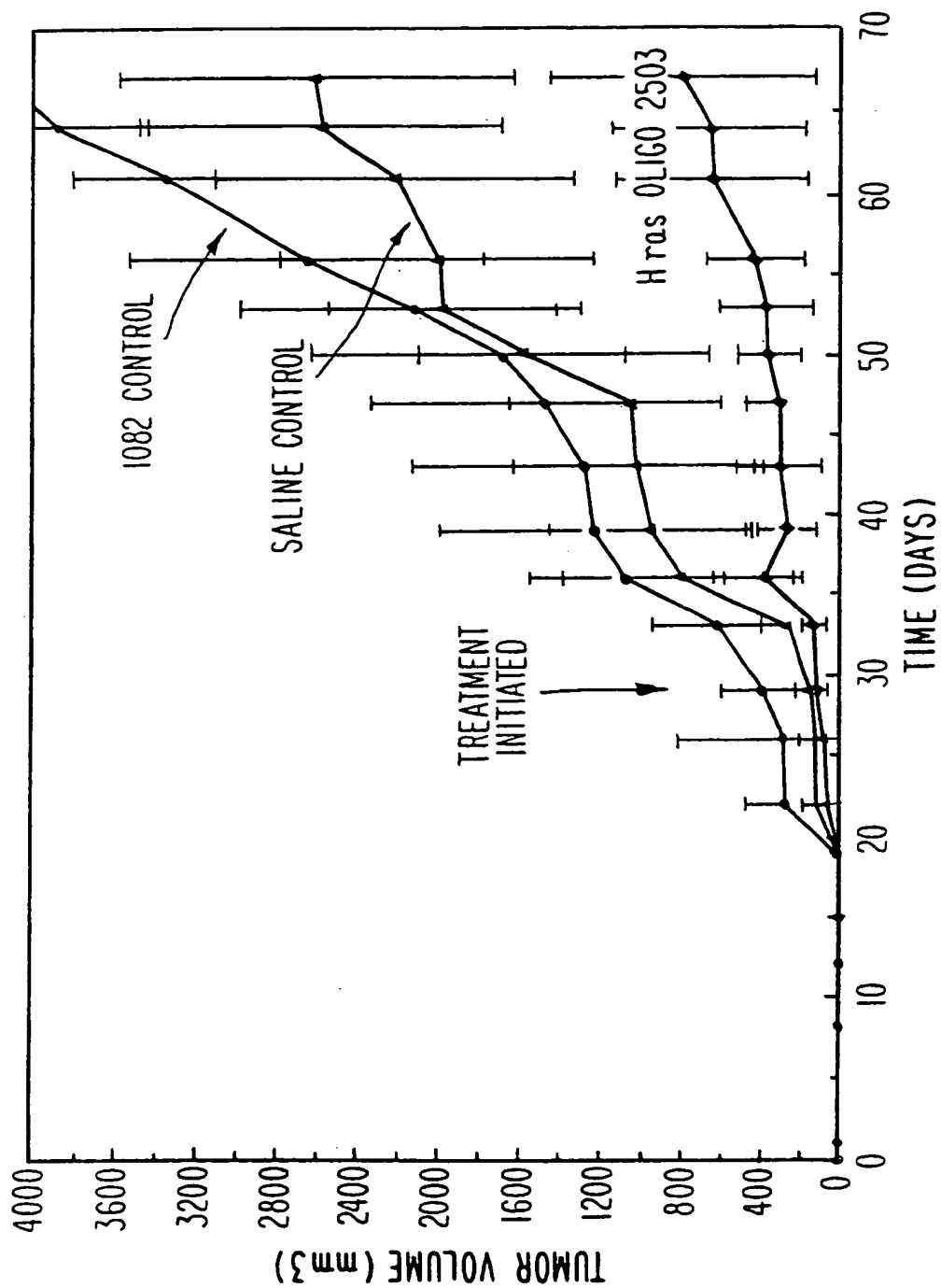
Fig. 1a***Fig. 1b******Fig. 1c***

Fig. 1d***Fig. 1e******Fig. 1f***

**Fig. 2**

***Fig. 1g******Fig. 1h***

**Fig. 4**

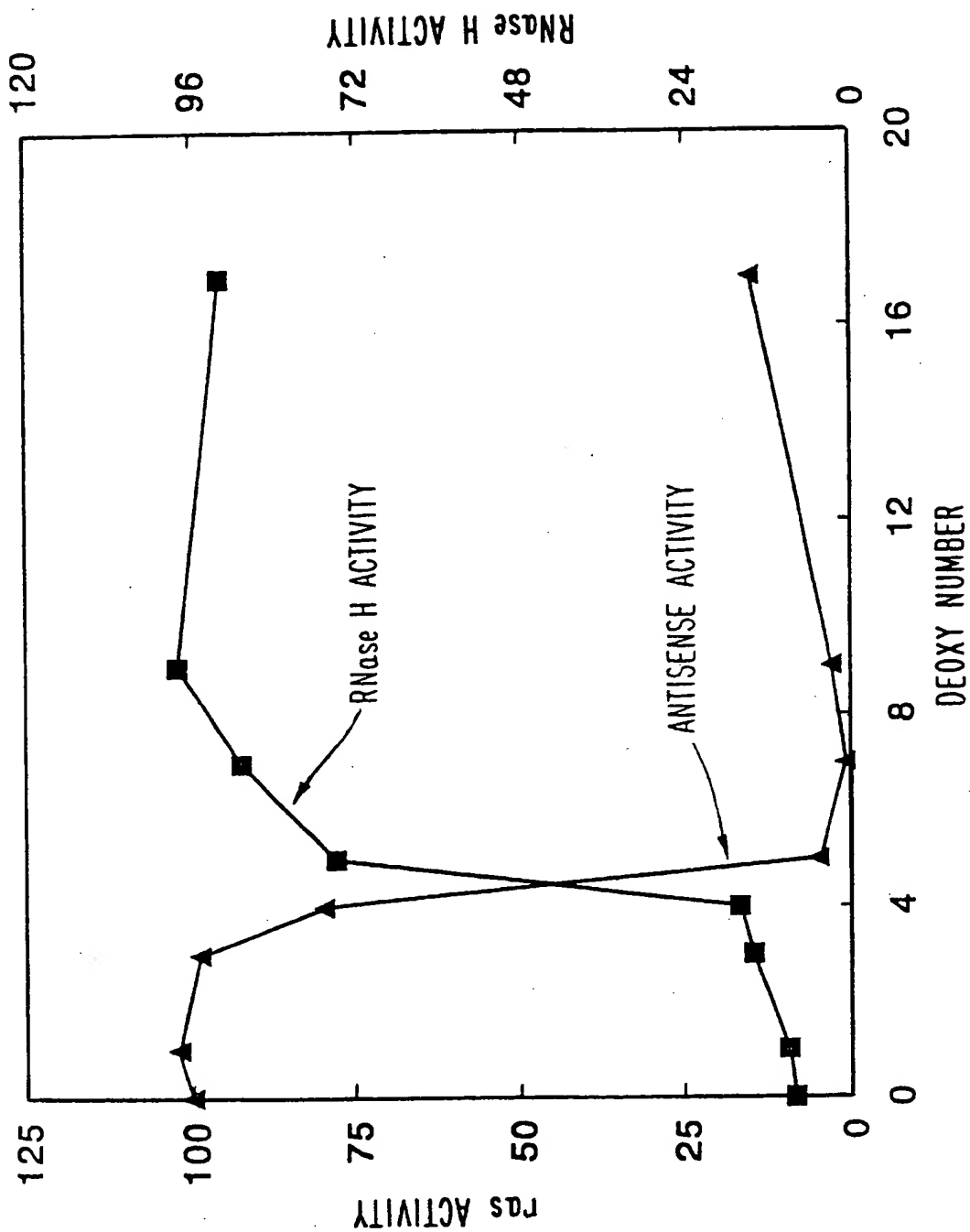


Fig. 3

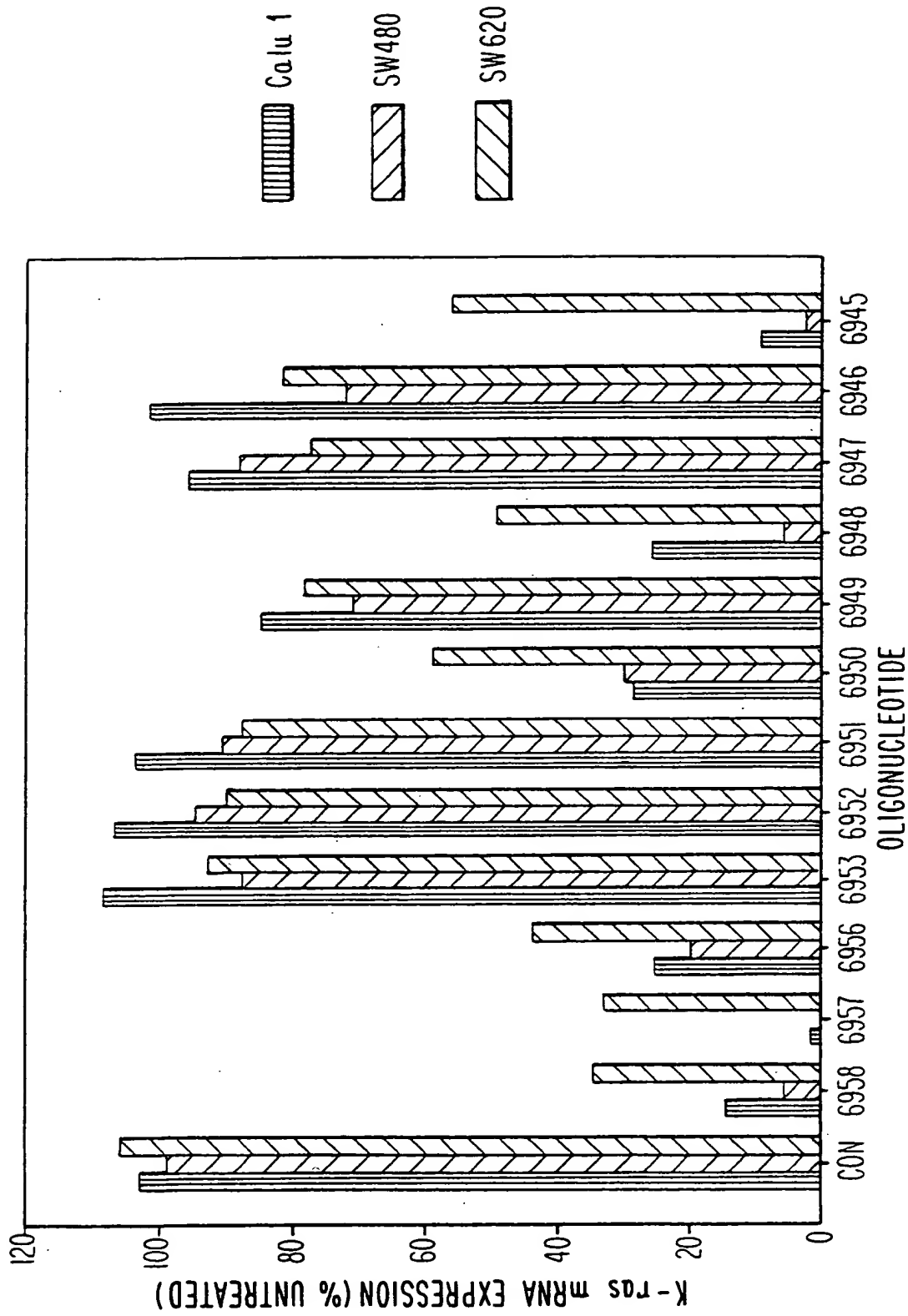
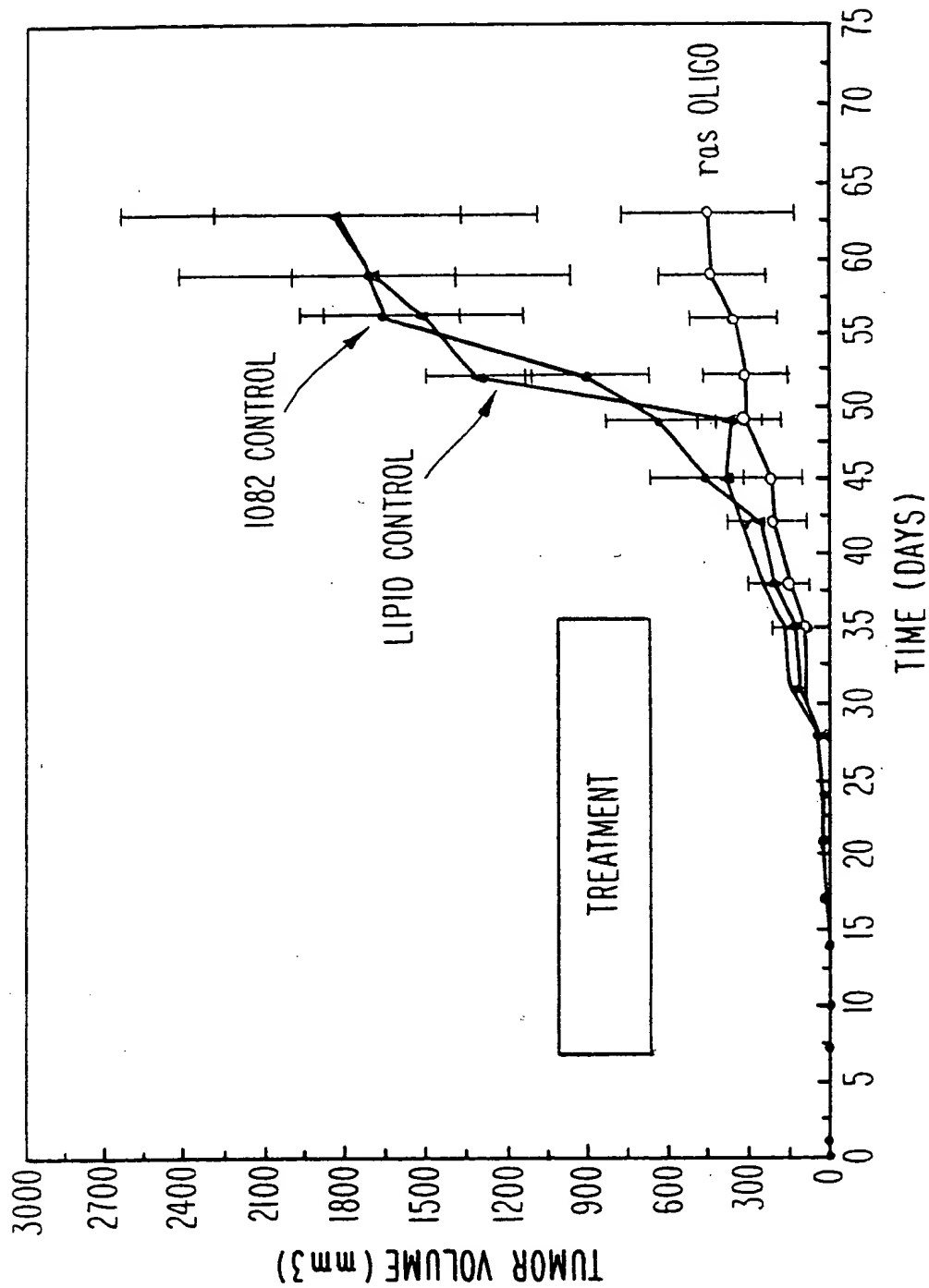
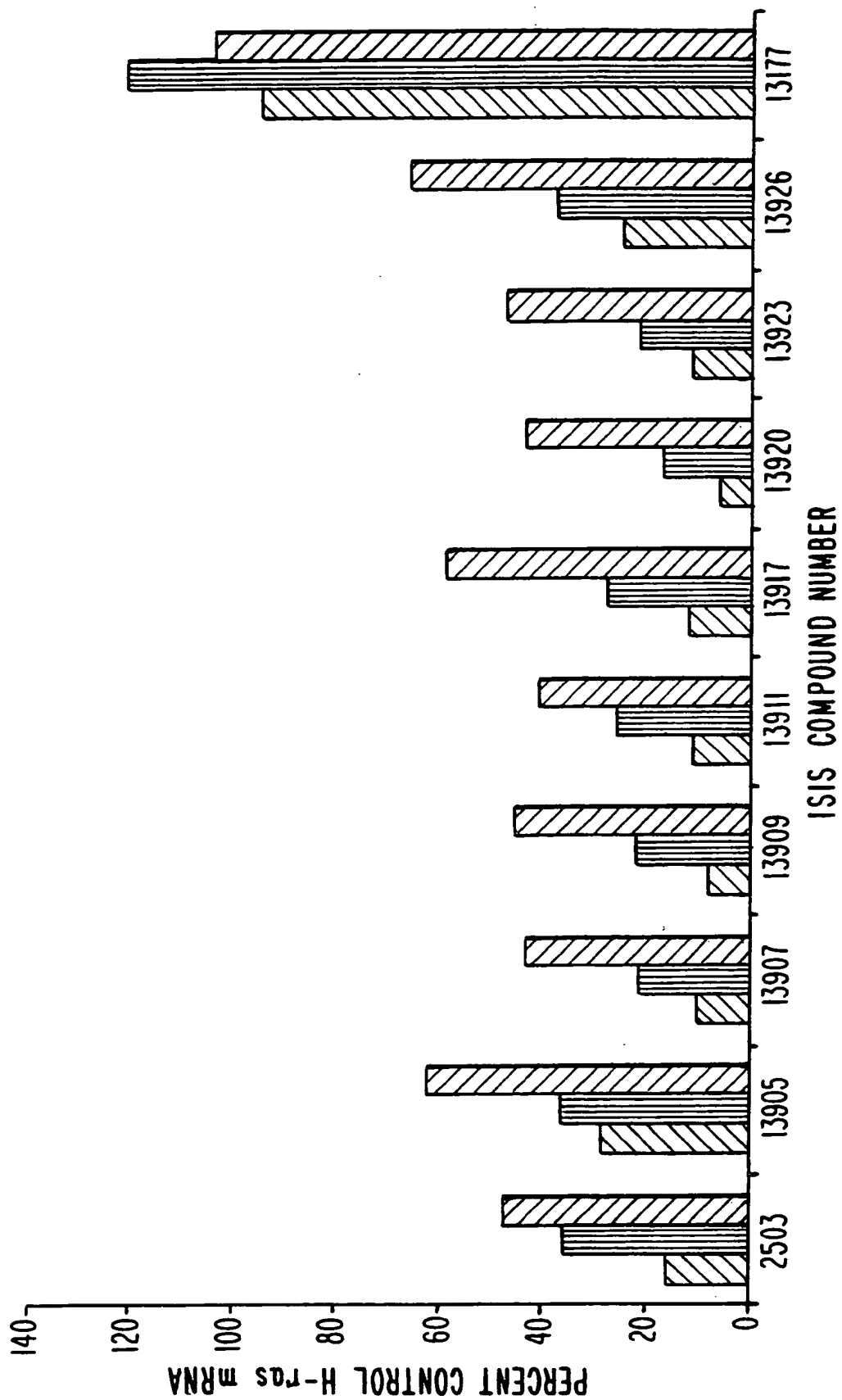


Fig. 6

**Fig. 5**

**Fig. 8**

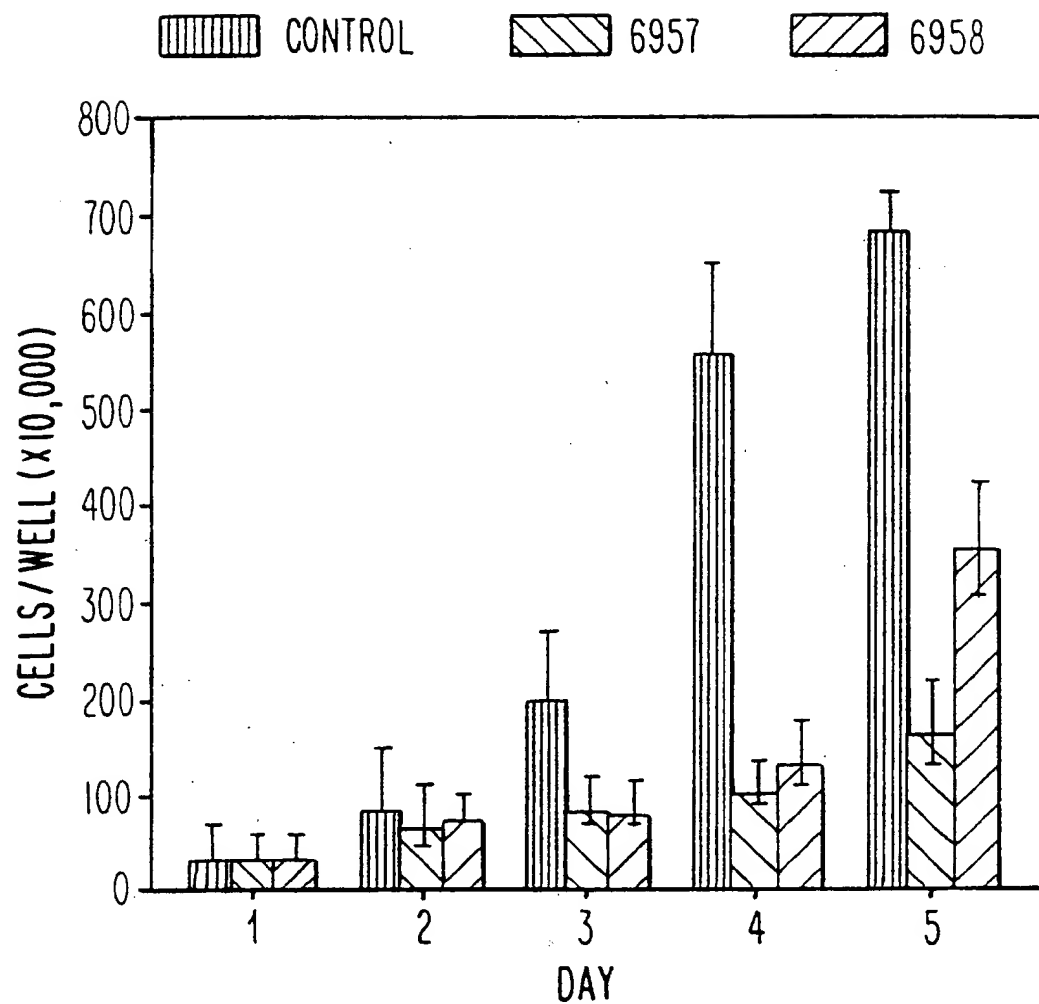
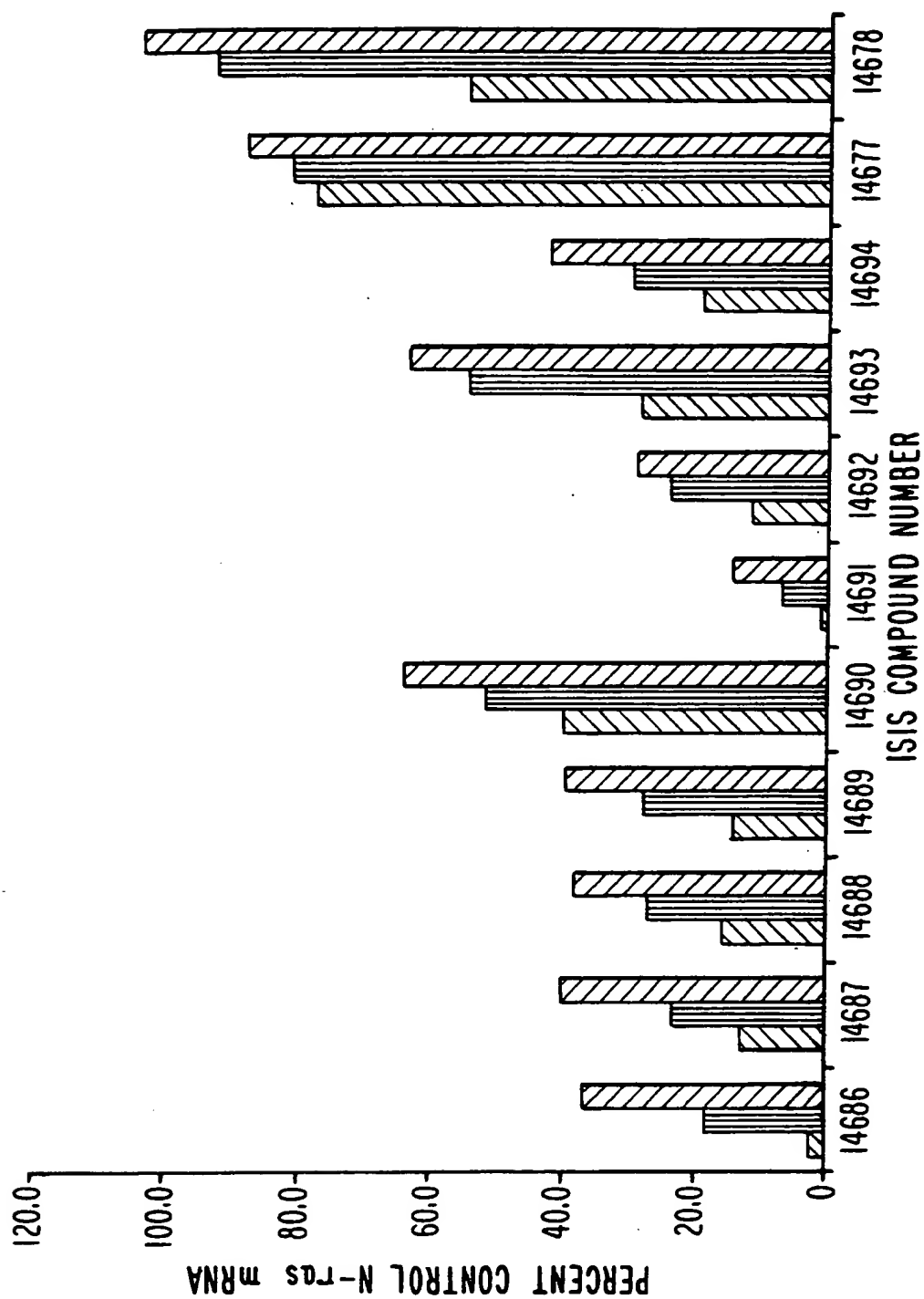
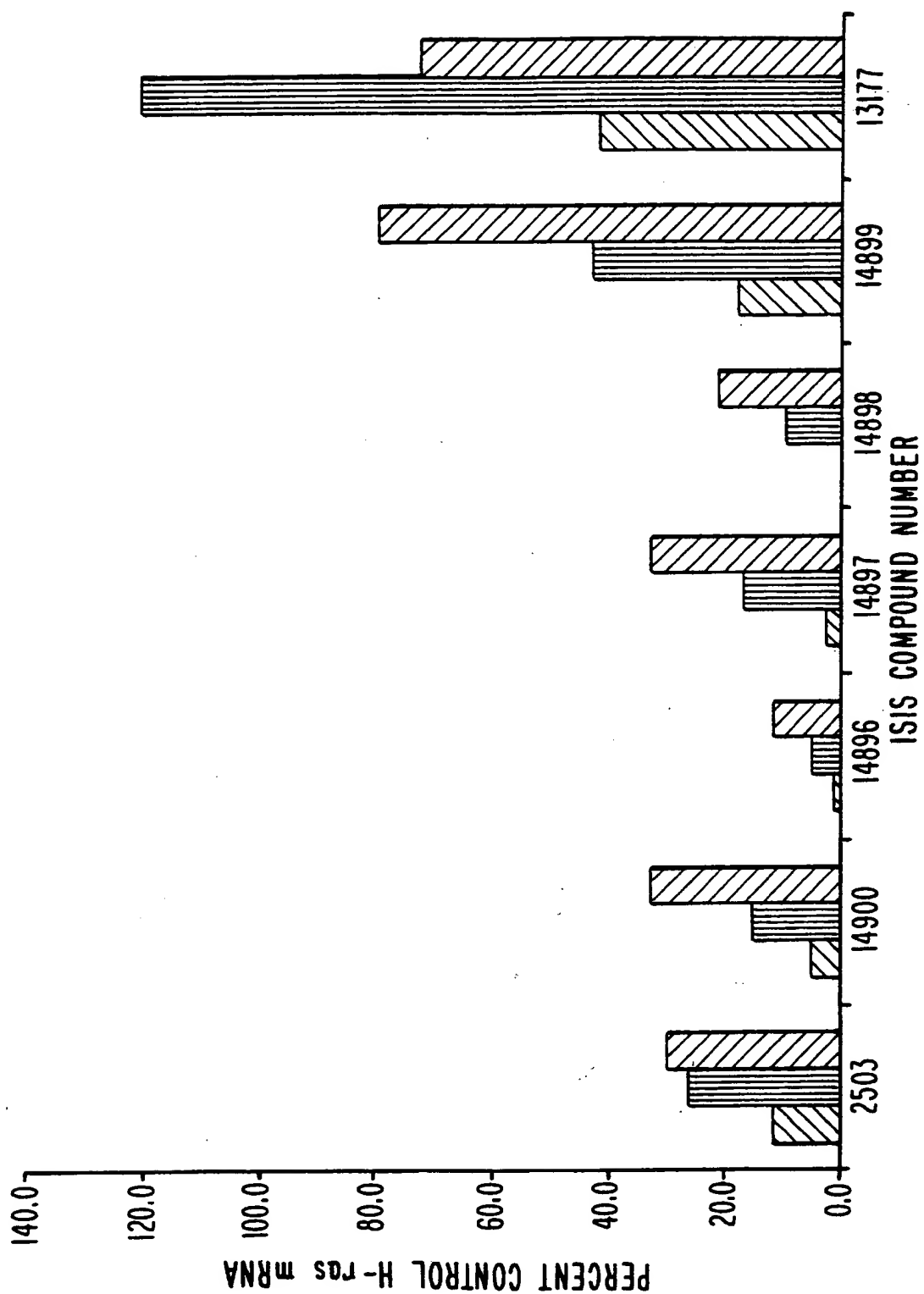


Fig. 7

*Fig. 10*

**Fig. 9**



US006117848A

United States Patent [19]**Monia et al.**[11] **Patent Number:** **6,117,848**[45] **Date of Patent:** ***Sep. 12, 2000**[54] **ANTISENSE OLIGONUCLEOTIDE
INHIBITION OF RAS**[75] **Inventors:** **Brett P. Monia, La Costa; Lex M. Cowser; Muthiah Manoharan**, both of Carlsbad, all of Calif.[73] **Assignee:** **Isis Pharmaceuticals Inc.**, Carlsbad, Calif.[*] **Notice:** This patent is subject to a terminal disclaimer.[21] **Appl. No.:** **09/128,494**[22] **Filed:** **Aug. 3, 1998****Related U.S. Application Data**

[63] Continuation-in-part of application No. 08/411,734, filed as application No. PCT/US93/09346, Oct. 1, 1993, which is a continuation-in-part of application No. 07/958,134, Oct. 5, 1992, abandoned, and a continuation-in-part of application No. 08/007,996, Jan. 21, 1993, abandoned.

[51] **Int. Cl.⁷** **A61K 48/00; C12Q 1/68; C07H 21/02; C07H 21/04**[52] **U.S. Cl.** **514/44; 435/6; 435/91.2; 536/23.1; 536/24.3; 536/24.5**[58] **Field of Search** **435/6, 91.2; 536/23.1; 536/24.3, 24.5; 514/44**[56] **References Cited****U.S. PATENT DOCUMENTS**

4,871,838	10/1989	Bos et al.	536/27
5,034,506	7/1991	Summerton et al.	528/391
5,138,045	8/1992	Cook et al.	536/27
5,218,105	6/1993	Cook et al.	536/25.31
5,378,825	1/1995	Cook et al.	536/25.34
5,459,255	10/1995	Cook et al.	536/27.13
5,576,208	11/1996	Monia et al.	435/240.2
5,582,986	12/1996	Monia et al.	435/6
5,623,065	4/1997	Cook et al.	536/23.1

FOREIGN PATENT DOCUMENTS

PCT/US88/		
01024	3/1988	WIPO.
WO 92/15680	9/1992	WIPO.
WO 94/26764	11/1994	WIPO.

OTHER PUBLICATIONSGewirtz et al., "Facilitating oligonucleotide delivery: helping antisense deliver on its promise", *Proc. Natl. Acad. Sci.* 93:3161-3163, Apr. 1996.Stein, C., "Keeping the biotechnology of antisense in context", *Nature Biotechnology* 17:209, Mar. 1999.Anfossi et al., "An Oligomer Complementary to c-myc encoded mRNA inhibits proliferation of human myeloid leukemia cell lines", *Proc. Natl. Acad. Sci.* 1989, 86, 3379-3383.Borer, P.N., Dengler, B., Tinoco, I., Jr., and Uhlenbeck, O.C., "Stability of Ribonucleic acid Double-stranded Helices", *J. Mol. Biol.*, 1974, 86, 843-853.Brown et al., "Modulation of ras Expression by Antisense, Nonionic Deoxyoligonucleotide Analogs", *Oncogene Research* 1989, 4, 243-252.Capon et al., "Complete nucleotide sequence of the T24 human bladder carcinoma oncogene and its normal homologue", *Nature* 302 1983, 33-37.Chang et al., "Comparative inhibition of ras p21 protein synthesis with phosphorus-modified antisense oligonucleotides", *Anti-Cancer Drug Design* 1989, 4, 221-232.Chang et al., "Antisense inhibition of ras p21 expression that is sensitive to a point mutation", *Biochemistry* 1991, 30, 8283-8286.Chiang et al., "Antisense Oligonucleotides Inhibit Intercellular Adhesion Molecule 1 Expression by Two Distinct Mechanisms", *J. Biol. Chem.* 1991, 266:18162-18171.De Mesmaeker et al., "Antisense Oligonucleotides", *Acc. Chem. Res.* 1995, 28:366-374.Dignam et al., "Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei", *Nucleic Acids Res.* 1983, 11, 1475-1489.Feramisco et al., "Transient reversion of ras oncogene-induced cell transformation by antibodies specific for amino acid 12 of ras protein", *Nature* 1985, 314, 639-642.Gebeyehu, G., et al., "Novel biotinylated nucleotide-analogs for labeling and colorimetric detection of DNA", *Nucl. Acids Res.* 1987, 15:4513-4534.Georges, R.N. et al., "Prevention of Orthotopic Human Lung Cancer Growth by Intratracheal Instillation of a Retroviral Antisense K-ras Construct", 1993, *Cancer Research*, 53, 1743-1746.Greenberg, M.E., in *Current Protocols in Molecular Biology*, (F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.A. Smith, J.G. Seidman and K. Strahl, eds.), John Wiley and Sons, NY Undated.Hall et al., "Identification of transforming gene in two human sarcoma cell lines as a new member of the ras gene family located on chromosome 1", *Nature* 1983, 303: 396-400.Hall and Brown, "Human N-ras: cDNA cloning and gene structure", *Nucleic Acids Res.* 1985, 13, 5255-5268.Holt et al., "An Oligomer Complementary to c-myc mRNA Inhibits Proliferation of HL-60 Promyelocytic Cells and Induces Differentiation", *Mol. Cell Biol.* 1988, 8, 963-973.

(List continued on next page.)

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[57]

ABSTRACT

Compositions and methods are provided for the modulation of ras expression. Oligonucleotides are provided which are targeted to nucleic acids encoding human ras. Oligonucleotides specifically hybridizable with mRNA encoding human H-ras, Ki-ras and N-ras are provided. Such oligonucleotides can be used for therapeutics and diagnostics as well as for research purposes. Methods are also disclosed for modulating ras gene expression in cells and tissues using the oligonucleotides provided, and for specific modulation of expression of activated ras. Methods for diagnosis, detection and treatment of conditions associated with ras are also disclosed.

16 Claims, 12 Drawing Sheets

OTHER PUBLICATIONS

- Kabanov et al., "A new class of antivirals: antisense oligonucleotides combined with a hydrophobic substituent effectively inhibit influenza virus reproduction and synthesis of virus-specific proteins in MDCK cells", *FEBS Lett.* 1990, 259, 327-330.
- Kahn et al., "The c-K-ras gene and human cancer (review)", *Anticancer Res.* 1987, 7, 639-652.
- Kawasaki et al., "Uniformly Modified 2'-Deoxy-2'-fluoro Phosphorothioate Oligonucleotides as Nuclease-Resistant Antisense Compounds with High Affinity and Specificity for RNA Targets", *J. Med. Chem.* 1993, 36, 831-841.
- Kingston, R.E., in *Current Protocols in Molecular Biology*, (F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.A. Smith, J.G. Seidman and K. Strahl, eds.), John Wiley and Sons, NY, Undated.
- Kornberg, A., *DNA Replication*, W.H. Freeman & Co., San Francisco, 1980, pp 75-77.
- Lima et al., "Implication of RNA Structure on Antisense Oligonucleotide Hybridization Kinetics", *Biochemistry* 1992, 31, 12055-12061.
- Letsinger et al., "Cholesteryl-conjugated oligonucleotides: Synthesis, properties, and activity as inhibitors of replication of human immunodeficiency virus in cell culture", *Proc. Natl. Acad. Sci. USA* 1989, 86, 6553-6556.
- Mukhopadhyay, T. et al., "Specific Inhibition of K-ras Expression and Tumorigenicity of Lung Cancer Cells by Antisense RNA", (1991) *Cancer Research* 51, 1744-1748.
- Martin, P., "Ein neuer Zugang zu 2'-O-Alkylribonucleosiden und Eigenschaften deren Oligonucleotide", *Helv. Chim. Acta* 1995, 78, 486-504.
- Manoharan et al., "Cholic Acid-Oligonucleotide Conjugates for Antisense Applications", *Bioorg. Med. Chem. Lett.* 1994, 4, 1053-1060.
- Manoharan et al., "Introduction of a Lipophilic Thioether Tether in the Minor Groove of Nucleic Acids for Antisense Applications", *Bioorg. Med. Chem. Lett.* 1993, 3, 2765-2770.
- Manoharan et al., "Antisense Strategies", *Ann. N.Y. Acad. Sci.* 1992, 660, 306-309.
- Manoharan et al., "Lipidic Nucleic Acids", *Tetrahedron Lett.* 1995, 36, 3651-3654.
- Manoharan et al., "Oligonucleotides Conjugates: Alteration of the Pharmacokinetic Properties of Antisense Agents", *Nucleosides & Nucleotides* 1995, 14, 969-973.
- McGrath, J.P. et al., "Structure and organization of the human Ki-ras proto-oncogene and a related processed pseudogene", *Nature* 1983, 304, 501-506.
- Nielsen et al., "Sequence-Selective Recognition of DNA by Strand Displacement with a Thymine-Substituted Polyamide", *Science* 1991, 254, 1497.
- Oberhauser et al., "Effective incorporation of 2'-O-methyl-oligoribonucleotides into liposomes and enhanced cell association through modification with thiocholesterol", *Nucl. Acids Res.* 1992, 20, 533-538.
- Owen et al., "Transcriptional activation of a conserved sequence element by ras requires a nuclear factor distinct from c-fos or c-jun", *Proc. Natl. Acad. Sci. U.S.A.* 1990, 87, 3866-3870.
- Petersheim, M. and Turner, D.H., "Base Stacking and Base-Pairing Contributions to Helix Stability: Thermodynamics of Double-Helix Formation with CCGG, CCGGp, CCGGAp, ACCGGp, CCGGUp, and ACCGGUp", *Biochemistry* 1983, 22, 256-263.
- Puglisi and Tinoco, "Absorbance Melting Curves of RNAs", *Methods in Enzymol.* 1989, 180, 304-325.
- Reddy, P.E. et al., "A point mutation is responsible for the acquisition of transforming properties by the T24 human bladder carcinoma oncogene", *Nature* 1982, 300, 149-152.
- Sanghvi et al., "Antisense oligodeoxynucleotides: synthesis; biophysical and biological evaluation of oligodeoxynucleotides containing modified pyrimidines", *Nucl. Acids Res.* 1993, 21, 3197-3203.
- Sanghvi, Y.S., in Crooke, S.T. and Lebleu, B., eds., *Antisense Research and Applications*, CRC Press, Boca Raton, 1993, pp. 276-288.
- Saison-Behmoaras, T. et al., "Short modified antisense oligonucleotides directed against Ha-ras point mutation induce selective cleavage of the mRNA and inhibit T24 cells proliferation", *EMBO J.* 1991, 10, 1111-1118.
- Skorski, et al., "Growth Factor-dependent Inhibition of Normal Hematopoiesis by N-ras Antisense Oligodeoxynucleotides", *J. Exp. Med.*, 1992, 175, 743-750.
- Svinarchuk et al., "Inhibition of HIV proliferation in MT-4 cells by antisense oligonucleotide conjugated to lipophilic groups", *Biochimie* 1993, 75, 49-54.
- Shea, "Synthesis, hybridization properties and antiviral activity of lipid-oligodeoxynucleotide conjugates", et al. *Nucl. Acids Res.* 1990, 18, 3777-3783.
- Tidd et al., "Evaluation of N-ras oncogene anti-sense, sense and nonsense sequence methylphosphonate oligonucleotide analogues", *Anti-Cancer Drug Design* 1988, 3, 117-127.
- Tabin, C.J. et al., "Mechanism of activation of a human oncogene", *Nature* 1982, 300, 143-149.
- Taparowsky, E. et al., "Activation of the T24 bladder carcinoma transforming gene is linked to a single amino acid change", *Nature* 1982, 300, 762-765.
- Taparowsky et al., "Structure and Activation of the Human N-ras Gene", *Cell* 1983 34: 581-6.
- Wickstrom et al., "Human promyelocytic leukemia HL-60 cell proliferation and c-myc protein expression are inhibited by an antisense pentadecadeoxynucleotide targeted against c-myc mRNA", *Proc. Nat. Acad. Sci.* 1988, 85, 1028-1032.

ANTISENSE OLIGONUCLEOTIDE INHIBITION OF RAS

This application is a continuation-in-part of U.S. patent application Ser. No. 08/411,734, filed Apr. 3, 1995 which is a U.S. national phase application of PCT/US93/09346, filed Oct. 1, 1993, which is a continuation-in-part and foreign filing of U.S. patent application Ser. No. 07/958,134, filed Oct. 5, 1992, now abandoned and U.S. patent application Ser. No. 08/007,996, filed Jan. 21, 1993, now abandoned all of which are assigned to the assignee of the present invention and are incorporated herein by reference in their entirety.

FIELD OF THE INVENTION

This invention relates to compositions and methods for the inhibition of expression of ras, a naturally occurring protein which occasionally converts to an activated form that has been implicated in tumor formation. Antisense oligonucleotides targeted to H-, Ki- and N-ras are provided. This invention is further directed to the detection of both normal and activated forms of the ras gene in cells and tissues, and can form the basis for research reagents and kits both for research and diagnosis. Furthermore, this invention is directed to prevention and treatment of conditions associated with ras.

BACKGROUND OF THE INVENTION

Alterations in the cellular genes which directly or indirectly control cell growth and differentiation are considered to be the main cause of cancer. There are some thirty families of genes, called oncogenes, which are implicated in human tumor formation. Members of one such family, the ras gene family, are frequently found to be mutated in human tumors. In their normal state, proteins produced by the ras genes are thought to be involved in normal cell growth and maturation. Mutation of the ras gene, causing an amino acid alteration at one of three critical positions in the protein product, results in conversion to a form which is implicated in tumor formation. A gene having such a mutation is said to be "mutant" or "activated." Unmutated ras is called "wild-type" or "normal" ras. It is thought that such a point mutation leading to ras activation can be induced by carcinogens or other environmental factors. Over 90% of pancreatic adenocarcinomas, about 50% of adenomas and adenocarcinomas of the colon, about 50% of adenocarcinomas of the lung and carcinomas of the thyroid, and a large fraction of malignancies of the blood such as acute myeloid leukemia and myelodysplastic syndrome have been found to contain activated ras oncogenes. Overall, some 10 to 20% of human tumors have a mutation in one of the three ras genes (H-ras, Ki-ras, or N-ras).

It is presently believed that inhibiting expression of activated oncogenes in a particular tumor cell might force the cell back into more normal growth. For example, Feramisco et al., *Nature* 1985, 314, 639-642, demonstrated that if cells transformed to a malignant state with an activated ras gene are microinjected with antibody which binds to the protein product of the ras gene, the cells slow their rate of proliferation and adopt a more normal appearance. This has been interpreted as support for the involvement of the product of the activated ras gene in the uncontrolled growth typical of cancer cells.

There is a great desire to provide compositions of matter which can modulate the expression of ras, and particularly to provide compositions of matter which specifically modu-

late the expression of activated ras. It is greatly desired to provide methods of diagnosis and detection of nucleic acids encoding ras in animals. It is also desired to provide methods of diagnosis and treatment of conditions arising from ras activation. In addition, improved research kits and reagents for detection and study of nucleic acids encoding ras are desired.

Inhibition of oncogene expression has been accomplished using retroviral vectors or plasmid vectors which express a 2-kilobase segment of the Ki-ras protooncogene RNA in antisense orientation. Mukhopadhyay, T. et al. (1991) *Cancer Research* 51, 1744-1748; PCT Patent Application PCT/US92/01852 (WO 92/15680); Georges, R. N. et al. (1993) *Cancer Research*, 53, 1743-1746.

Antisense oligonucleotide inhibition of oncogenes has proven to be a useful tool in understanding the roles of various oncogene families. Antisense oligonucleotides are small oligonucleotides which are complementary to the "sense" or coding strand of a given gene, and as a result are also complementary to, and thus able to stably and specifically hybridize with, the mRNA transcript of the gene. Holt et al., *Mol. Cell Biol.* 1988, 8, 963-973, have shown that antisense oligonucleotides hybridizing specifically with mRNA transcripts of the oncogene c-myc, when added to cultured HL60 leukemic cells, inhibit proliferation and induce differentiation. Anfossi et al., *Proc. Natl. Acad. Sci.* 1989, 86, 3379-3383, have shown that antisense oligonucleotides specifically hybridizing with mRNA transcripts of the c-myc oncogene inhibit proliferation of human myeloid leukemia cell lines. Wickstrom et al., *Proc. Nat. Acad. Sci.* 1988, 85, 1028-1032, have shown that expression of the protein product of the c-myc oncogene as well as proliferation of HL60 cultured leukemic cells are inhibited by antisense oligonucleotides hybridizing specifically with c-myc mRNA. U.S. Pat. No. 4,871,838 (Bos et al.) discloses oligonucleotides complementary to a mutation in codon 13 of N-ras to detect said mutation. U.S. Pat. No. 4,871,838 (Bos et al.) discloses molecules useful as probes for detecting a mutation in DNA which encodes a ras protein.

In all these cases, instability of unmodified oligonucleotides has been a major problem, as they are subject to degradation by cellular enzymes. PCT/US88/01024 (Zon et al.) discloses phosphorothioate oligonucleotides hybridizable to the translation initiation region of the amplified c-myc oncogene to inhibit HL-60 leukemia cell growth and DNA synthesis in these cells. Tidd et al., *Anti-Cancer Drug Design* 1988, 3, 117-127, evaluated methylphosphonate antisense oligonucleotides hybridizing specifically to the activated N-ras oncogene and found that while they were resistant to biochemical degradation and were nontoxic in cultured human HT29 cells, they did not inhibit N-ras gene expression and had no effect on these cells. Chang et al. showed that both methylphosphonate and phosphorothioate oligonucleotides hybridizing specifically to mRNA transcripts of the mouse Balb-ras gene could inhibit translation of the protein product of this gene in vitro. Chang et al., *Anti-Cancer Drug Design* 1989, 4, 221-232; Brown et al., *Oncogene Research* 1989, 4, 243-252. It was noted that T_m was not well correlated with antisense activity of these oligonucleotides against in vitro translation of the ras p21 protein product. Because the antisense oligonucleotides used by Chang et al. hybridize specifically with the translation initiation region of the ras gene, they are not expected to show any selectivity for activated ras and the binding ability of these oligonucleotides to normal (wild-type) vs. mutated (activated) ras genes was not compared.

Helene and co-workers have demonstrated selective inhibition of activated (codon 12 G→T transition) H-ras mRNA

expression using a 9-mer phosphodiester linked to an acridine intercalating agent and/or a hydrophobic tail. This compound displayed selective targeting of mutant ras message in both Rnase H and cell proliferation assays at low micromolar concentrations. Saison-Behmoaras, T. et al., *EMBO J.* 1991, 10, 1111-1118. Chang and co-workers disclose selective targeting of mutant H-ras message; this time the target was H-ras codon 61 containing an A→T transversion and the oligonucleotide employed was either an 11-mer methylphosphonate or its psoralen derivative. These compounds, which required concentrations of 7.5-150 μ M for activity, were shown by immunoprecipitation to selectively inhibit mutant H-ras p21 expression relative to normal p21. Chang et al., *Biochemistry* 1991, 30, 8283-8286.

SUMMARY OF THE INVENTION

The present invention relates to antisense oligonucleotides which are targeted to human ras, and methods of using them. More specifically, the present invention provides oligonucleotides which are targeted to mRNA encoding human H-ras, Ki-ras and N-ras and which are capable of inhibiting ras expression. oligonucleotides targeted to a 5' untranslated region, translation initiation site, coding region or 3' untranslated region of human N-ras are provided. Methods of modulating ras expression, of inhibiting the proliferation of cancer cells and of treating conditions associated with ras are provided. These methods employ the oligonucleotides of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a series of 8 panels showing inhibition of ras in a dose-dependent manner. Solid lines are activity against wild-type (normal) ras, dotted lines show activity against activated (mutant) ras.

FIG. 2 is a bar graph showing antisense activities of a uniform deoxy phosphorothioate and shortened chimeric oligonucleotides against ras-luciferase.

FIG. 3 is a line graph showing correlation between antisense activity and ability to activate RNase H as a function of deoxy gap length using phosphorothioate 2'-O-methyl oligonucleotides targeted against ras.

FIG. 4 is a line graph showing anti-tumor activity of ISIS 2503 against A549 human cell tumors in nude mice.

FIG. 5 is a line graph showing anti-tumor activity of ras oligo ISIS 2503, administered with cationic lipid, against A549 human cell tumors in nude mice.

FIG. 6 is a bar graph showing antisense inhibition of Ki-ras mRNA expression in three human colon carcinoma cell lines, Calu1, SW480 and SW620.

FIG. 7 is a bar graph showing inhibition of SW480 human carcinoma cell line proliferation by Ki-ras specific oligonucleotides ISIS 6957 and ISIS 6958.

FIG. 8 is a bar graph showing reduction of H-ras mRNA levels by 2'-MOE analogs of ISIS 2503 (SEQ ID NO: 2). Black bars: 150 nM oligonucleotide dose; Diagonal hatched bars: 50 nM dose; horizontal hatched bars: 15 nM dose.

FIG. 9 is a bar graph showing reduction of H-ras mRNA levels by MMI analogs of ISIS 2503 (SEQ ID NO: 2). Black bars: 500 nM oligonucleotide dose; Diagonal hatched bars: 100 nM dose; horizontal hatched bars: 50 nM dose.

FIG. 10 is a bar graph showing reduction of N-ras mRNA levels by oligonucleotides 14686-14694, 14677 and 14678. Black bars: 400 nM oligonucleotide dose; Diagonal hatched bars: 200 nM dose; horizontal hatched bars: 100 nM dose.

DETAILED DESCRIPTION OF THE INVENTION

Malignant tumors develop through a series of stepwise, progressive changes that lead to the loss of growth control

characteristic of cancer cells, i.e., continuous unregulated proliferation, the ability to invade surrounding tissues, and the ability to metastasize to different organ sites. Carefully controlled in vitro studies have helped define the factors that characterize the growth of normal and neoplastic cells and have led to the identification of specific proteins that control cell growth and differentiation. In addition, the ability to study cell transformation in carefully controlled, quantitative in vitro assays has led to the identification of specific genes capable of inducing the transformed cell phenotype. Such cancer-causing genes, or oncogenes, are believed to acquire transformation-inducing properties through mutations leading to changes in the regulation of expression of their protein products. In some cases such changes occur in non-coding DNA regulatory domains, such as promoters and enhancers, leading to alterations in the transcriptional activity of oncogenes, resulting in over- or under-expression of their gene products. In other cases, gene mutations occur within the coding regions of oncogenes, leading to the production of altered gene products that are inactive, overactive, or exhibit an activity that is different from the normal (wild-type) gene product.

To date, more than 30 cellular oncogene families have been identified. These genes can be categorized on the basis of both their subcellular location and the putative mechanism of action of their protein products. The ras oncogenes are members of a gene family which encode related proteins that are localized to the inner face of the plasma membrane. ras proteins have been shown to be highly conserved at the amino acid level, to bind GTP with high affinity and specificity, and to possess GTPase activity. Although the cellular function of ras gene products is unknown, their biochemical properties, along with their significant sequence homology with a class of signal-transducing proteins known as GTP binding proteins, or G proteins, suggest that ras gene products play a fundamental role in basic cellular regulatory functions relating to the transduction of extracellular signals across plasma membranes.

Three ras genes, designated H-ras, Ki-ras, and N-ras, have been identified in the mammalian genome. Mammalian ras genes acquire transformation-inducing properties by single point mutations within their coding sequences. Mutations in naturally occurring ras oncogenes have been localized to codons 12, 13, and 61. The sequences of H-ras, Ki-ras and N-ras are known. Capon et al., *Nature* 302 1983, 33-37; Kahn et al., *Anticancer Res.* 1987, 7, 639-652; Hall and Brown, *Nucleic Acids Res.* 1985, 13, 5255-5268. The most commonly detected activating ras mutation found in human tumors is in codon 12 of the H-ras gene in which a base change from GGC to GTC results in a glycine-to-valine substitution in the GTPase regulatory domain of the ras protein product. Tabin, C. J. et al., *Nature* 1982, 300, 143-149; Reddy, P. E. et al., *Nature* 1982, 300, 149-152; Taparowsky, E. et al., *Nature* 1982, 300, 762-765. This single amino acid change is thought to abolish normal control of ras protein function, thereby converting a normally regulated cell protein to one that is continuously active. It is believed that such deregulation of normal ras protein function is responsible for the transformation from normal to malignant growth. It is therefore believed that inhibition of ras expression is useful in treatment and/or prevention of malignant conditions, i.e., cancer and other hyperproliferative conditions.

The H-ras gene has recently been implicated in a serious cardiac arrhythmia called long Q-T syndrome, a hereditary condition which often causes sudden death if treatment is not given immediately. Frequently, there are no symptoms

prior to the onset of the erratic heartbeat. Whether the H-ras gene is precisely responsible for long Q-T syndrome is unclear. However, there is an extremely high correlation between inheritance of this syndrome and the presence of a particular variant of the chromosome 11 region surrounding the H-ras gene. Therefore, the H-ras gene is a useful indicator of increased risk of sudden cardiac death due to the long Q-T syndrome.

N-ras was first identified as an oncogene in gene transfer experiments. Hall et al. *Nature* 1983, 303: 396-400. Its activation was characterized by Taparowsky et al. *Cell* 1983 34: 581-6. Activated N-ras is found in many hematologic neoplasms and solid tumors, suggesting a role for N-ras in the development or maintenance of hyperproliferative conditions.

The present invention provides oligonucleotides for inhibition of human ras gene expression. Such oligonucleotides specifically hybridize with selected DNA or mRNA deriving from a human ras gene. The invention also provides oligonucleotides for selective inhibition of expression of the mutant form of ras. This relationship between an oligonucleotide and its complementary nucleic acid target to which it hybridizes is commonly referred to as "antisense". "Targeting" an oligonucleotide to a chosen nucleic acid target, in the context of this invention, is a multistep process. The process usually begins with identifying a nucleic acid sequence whose function is to be modulated. This may be, for example, a cellular gene (or mRNA made from the gene) whose expression is associated with a particular disease state, or a foreign nucleic acid from an infectious agent. In the present invention, the target is a nucleic acid encoding ras; in other words, the ras gene or mRNA expressed from the ras gene. The targeting process also includes determination of a site or sites within the nucleic acid sequence for the oligonucleotide interaction to occur such that the desired effect—modulation of gene expression—will result. Once the target site or sites have been identified, oligonucleotides are chosen which are sufficiently complementary to the target, i.e., hybridize sufficiently well and with sufficient specificity, to give the desired modulation.

In the context of this invention "modulation" means either inhibition or stimulation. Inhibition of ras gene expression is presently the preferred form of modulation. This modulation can be measured in ways which are routine in the art, for example by Northern blot assay of mRNA expression or Western blot assay of protein expression as taught in the examples of the instant application. Effects on cell proliferation or tumor cell growth can also be measured, as taught in the examples of the instant application. "Hybridization", in the context of this invention, means hydrogen bonding, also known as Watson-Crick base pairing, between complementary bases, usually on opposite nucleic acid strands or two regions of a nucleic acid strand. Guanine and cytosine are examples of complementary bases which are known to form three hydrogen bonds between them. Adenine and thymine are examples of complementary bases which form two hydrogen bonds between them. "Specifically hybridizable" and "complementary" are terms which are used to indicate a sufficient degree of complementarity such that stable and specific binding occurs between the DNA or RNA target and the oligonucleotide. It is understood that an oligonucleotide need not be 100% complementary to its target nucleic acid sequence to be specifically hybridizable. An oligonucleotide is specifically hybridizable when binding of the oligonucleotide to the target interferes with the normal function of the target molecule to cause a loss of utility, and there is a sufficient degree of complementarity to

avoid non-specific binding of the oligonucleotide to non-target sequences under conditions in which specific binding is desired, i.e., under physiological conditions in the case of in vivo assays or therapeutic treatment or, in the case of in vitro assays, under conditions in which the assays are conducted.

In preferred embodiments of this invention, oligonucleotides are provided which are targeted to mRNA encoding H-ras, Ki-ras or N-ras. In accordance with this invention, persons of ordinary skill in the art will understand that mRNA includes not only the coding region which carries the information to encode a protein using the three letter genetic code, including the translation start and stop codons, but also associated ribonucleotides which form a region known to such persons as the 5'-untranslated region, the 3'-untranslated region, the 5' cap region, intron regions and intron/exon or splice junction ribonucleotides. Thus, oligonucleotides may be formulated in accordance with this invention which are targeted wholly or in part to these associated ribonucleotides as well as to the coding ribonucleotides. In preferred embodiments, the oligonucleotide is targeted to a translation initiation site (AUG codon) or sequences in the coding region, 5' untranslated region or 3'-untranslated region of the ras mRNA. The functions of messenger RNA to be interfered with include all vital functions such as translocation of the RNA to the site for protein translation, actual translation of protein from the RNA, splicing or maturation of the RNA and possibly even independent catalytic activity which may be engaged in by the RNA. The overall effect of such interference with the RNA function is to cause interference with ras protein expression.

The present invention provides oligonucleotides for modulation of ras gene expression. Such oligonucleotides are targeted to nucleic acids encoding ras. As hereinbefore defined, "modulation" means either inhibition or stimulation. Inhibition of ras gene expression is presently the preferred form of modulation.

In the context of this invention, the term "oligonucleotide" refers to an oligomer or polymer of nucleotide or nucleoside monomers consisting of naturally occurring bases, sugars and intersugar (backbone) linkages. The term "oligonucleotide" also includes oligomers comprising non-naturally occurring monomers, or portions thereof, which function similarly. Such modified or substituted oligonucleotides are often preferred over native forms because of properties such as, for example, enhanced cellular uptake and increased stability in the presence of nucleases.

Certain preferred oligonucleotides of this invention are chimeric oligonucleotides. "Chimeric oligonucleotides" or "chimeras", in the context of this invention, are oligonucleotides which contain two or more chemically distinct regions, each made up of at least one nucleotide. These oligonucleotides typically contain at least one region of modified nucleotides that confers one or more beneficial properties (such as, for example, increased nuclease resistance, increased uptake into cells, increased binding affinity for the RNA target) and a region that is a substrate for enzymes capable of cleaving RNA:DNA or RNA:RNA hybrids. By way of example, RNase H is a cellular endonuclease which cleaves the RNA strand of an RNA:DNA duplex. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing the efficiency of antisense inhibition of gene expression. Consequently, comparable results can often be obtained with shorter oligonucleotides when chimeric oligos are used, compared to phosphorothioate deoxyoligonucleotides hybridizing to the

same target region. Cleavage of the RNA target can be routinely detected by gel electrophoresis and, if necessary, associated nucleic acid hybridization techniques known in the art. In one preferred embodiment, a chimeric oligonucleotide comprises at least one region modified to increase target binding affinity, and, usually, a region that acts as a substrate for RNase H. Affinity of an oligonucleotide for its target (in this case, a nucleic acid encoding ras) is routinely determined by measuring the T_m of an oligonucleotide/target pair, which is the temperature at which the oligonucleotide and target dissociate; dissociation is detected spectrophotometrically. The higher the T_m , the greater the affinity of the oligonucleotide for the target. In a more preferred embodiment, the region of the oligonucleotide which is modified to increase ras mRNA binding affinity comprises at least one nucleotide modified at the 2' position of the sugar, most preferably a 2'-O-alkyl, 2'-O-alkyl-O-alkyl or 2'-fluoro-modified nucleotide. Such modifications are routinely incorporated into oligonucleotides and these oligonucleotides have been shown to have a higher T_m (i.e., higher target binding affinity) than 2'-deoxyoligonucleotides against a given target. The effect of such increased affinity is to greatly enhance antisense oligonucleotide inhibition of ras gene expression. RNase H is a cellular endonuclease that cleaves the RNA strand of RNA:DNA duplexes; activation of this enzyme therefore results in cleavage of the RNA target, and thus can greatly enhance the efficiency of antisense inhibition. Cleavage of the RNA target can be routinely demonstrated by gel electrophoresis. In another preferred embodiment, the chimeric oligonucleotide is also modified to enhance nuclease resistance. Cells contain a variety of exo- and endo-nucleases which can degrade nucleic acids. A number of nucleotide and nucleoside modifications have been shown to make the oligonucleotide into which they are incorporated more resistant to nuclease digestion than the native oligodeoxynucleotide. Nuclease resistance is routinely measured by incubating oligonucleotides with cellular extracts or isolated nuclease solutions and measuring the extent of intact oligonucleotide remaining over time, usually by gel electrophoresis. Oligonucleotides which have been modified to enhance their nuclease resistance survive intact for a longer time than unmodified oligonucleotides. A variety of oligonucleotide modifications have been demonstrated to enhance or confer nuclease resistance. Oligonucleotides which contain at least one phosphorothioate modification are presently more preferred. In some cases, oligonucleotide modifications which enhance target binding affinity are also, independently, able to enhance nuclease resistance. A discussion of antisense oligonucleotides and some desirable modifications can be found in De Mesmaeker et al. *Acc. Chem. Res.* 1995, 28:366-374.

Specific examples of some preferred oligonucleotides envisioned for this invention include those containing modified backbones, for example, phosphorothioates, phosphotriesters, methyl phosphonates, short chain alkyl or cycloalkyl intersugar linkages or short chain heteroatomic or heterocyclic intersugar linkages. Most preferred are oligonucleotides with phosphorothioate backbones and those with heteroatom backbones, particularly $\text{CH}_2\text{—NH—O—CH}_2$, $\text{CH}_2\text{—N(CH}_3\text{)—O—CH}_2$ [known as a methylene (methylimino) or MMI backbone], $\text{CH}_2\text{—O—N(CH}_3\text{)—CH}_2$, $\text{CH}_2\text{—N(CH}_3\text{)—N(CH}_3\text{)—CH}_2$ and $\text{O—N(CH}_3\text{)—CH}_2\text{—CH}_2$ backbones, wherein the native phosphodiester backbone is represented as O—P—O—CH_2 . The amide backbones disclosed by De Mesmaeker et al. *Acc. Chem. Res.* 1995, 28:366-374) are also preferred. Also preferred

are oligonucleotides having morpholino backbone structures (Summerton and Weller, U.S. Pat. No. 5,034,506). In other preferred embodiments, such as the peptide nucleic acid (PNA) backbone, the phosphodiester backbone of the oligonucleotide is replaced with a polyamide backbone, the nucleobases being bound directly or indirectly to the nitrogen atoms of the polyamide backbone (Nielsen et al. *Science* 1991, 254, 1497).

Oligonucleotides may also contain one or more substituted sugar moieties. Preferred oligonucleotides comprise one of the following at the 2' position: OH, SH, SCH_3 , F, OCN, OCH_2OCH_3 , $\text{OCH}_2\text{O(CH}_2\text{)}_n\text{CH}_3$, $\text{O(CH}_2\text{)}_n\text{NH}_2$ or $\text{O(CH}_2\text{)}_n\text{CH}_3$ where n is from 1 to about 10; C_1 to C_{10} lower alkyl, alkoxyalkoxy, substituted lower alkyl, alkaryl or aralkyl; Cl; Br; CN; CF_3 ; OCF_3 ; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; SOCH_3 ; SO_2CH_3 ; ONO_2 ; NO_2 ; N_3 ; NH_2 ; heterocycloalkyl; heterocycloalkaryl; aminoalkylamino; polyalkylamino; substituted silyl; an RNA cleaving group; a reporter group; an intercalator; a group for improving the pharmacokinetic properties of an oligonucleotide; or a group for improving the pharmacodynamic properties of an oligonucleotide and other substituents having similar properties. A preferred modification includes 2'-methoxyethoxy [2'-O— $\text{CH}_2\text{CH}_2\text{OCH}_3$, also known as 2'-O-(2-methoxyethyl)] (Martin et al., *Helv. Chim. Acta*, 1995, 78, 486). Other preferred modifications include 2'-methoxy (2'-O— CH_3), 2'-propoxy (2'-O— $\text{CH}_2\text{CH}_2\text{CH}_3$) and 2'-fluoro (2'-F). Similar modifications may also be made at other positions on the oligonucleotide, particularly the 3' position of the sugar on the 3' terminal nucleotide and the 5' position of 5' terminal nucleotide. Oligonucleotides may also have sugar mimetics such as cyclobutyls in place of the pentofuranosyl group.

Oligonucleotides may also include, additionally or alternatively, nucleobase (often referred to in the art simply as "base") modifications or substitutions. As used herein, "unmodified" or "natural" nucleobases include adenine (A), guanine (G), thymine (T), cytosine (C) and uracil (U). Modified nucleobases include nucleobases found only infrequently or transiently in natural nucleic acids, e.g., hypoxanthine, 6-methyladenine, 5-me pyrimidines, particularly 5-methylcytosine (also referred to as 5-methyl-2'-deoxycytosine and often referred to in the art as 5-me-C), 5-hydroxymethylcytosine (HMC), glycosyl HMC and gen-tobiosyl HMC, as well as synthetic nucleobases, e.g., 2-aminoadenine, 2-(methylamino)adenine, 2-(imidazolylalkyl)adenine, 2-(aminoalkylamino)adenine or other heterosubstituted alkyladenines, 2-thiouracil, 2-thiothymine, 5-bromouracil, 5-hydroxymethyluracil, 8-azaguanine, 7-deazaguanine, N^6 (6-aminoethyl)adenine and 2,6-diaminopurine. Kornberg, A., *DNA Replication*, W. H. Freeman & Co., San Francisco, 1980, pp75-77; Gebeyehu, G., et al. *Nucl. Acids Res.* 1987, 15:4513). A "universal" base known in the art, e.g., inosine, may be included. 5-me-C substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2° C. (Sanghvi, Y. S., in Crooke, S. T. and Lebleu, B., eds., *Antisense Research and Applications*, CRC Press, Boca Raton, 1993, pp. 276-278) and are presently preferred base substitutions.

Another modification of the oligonucleotides of the invention involves chemically linking to the oligonucleotide one or more moieties or conjugates which enhance the activity or cellular uptake of the oligonucleotide. Such moieties include but are not limited to lipid moieties such as a cholesterol moiety, a cholesteryl moiety (Letsinger et al., *Proc. Natl. Acad. Sci. USA* 1989, 86, 6553), cholic acid (Manoharan et al. *Bioorg. Med. Chem. Lett.* 1994, 4, 1053), a thioether, e.g., hexyl-S-tritylthiol (Manoharan et al. *Ann. N.Y. Acad. Sci.*

1992, 660, 306; Manoharan et al. *Bioorg. Med. Chem. Lett.* 1993, 3, 2765), a thiocholesterol (Oberhauser et al., *Nucl. Acids Res.* 1992, 20, 533), an aliphatic chain, e.g., dodecan-
diol or undecyl residues (Saison-Behmoaras et al. *EMBO J.* 1991, 10, 111; Kabanov et al. *FEBS Lett.* 1990, 259, 327; Svinarchuk et al. *Biochimie* 1993, 75, 49), a phospholipid,
e.g., di-hexadecyl-rac-glycerol or triethylammonium 1,2-di-
O-hexadecyl-rac-glycero-3-H-phosphonate (Manoharan et al. *Tetrahedron Lett.* 1995, 36, 3651; Shea et al. *Nucl. Acids Res.* 1990, 18, 3777), a polyamine or a polyethylene glycol
chain (Manoharan et al. *Nucleosides & Nucleotides* 1995, 14, 969), or adamantane acetic acid (Manoharan et al. *Tetrahedron Lett.* 1995, 36, 3651). Oligonucleotides com-
prising lipophilic moieties, and methods for preparing such
oligonucleotides are known in the art, for example, U.S. Pat.
Nos. 5,138,045, 5,218,105 and 5,459,255.

The oligonucleotides of the invention may be provided as
prodrugs, which comprise one or more moieties which are
cleaved off, generally in the body, to yield an active oligo-
nucleotide. One example of a prodrug approach is described
by Imbach et al. in WO Publication 94/26764.

It is not necessary for all positions in a given oligonucle-
otide to be uniformly modified, and in fact more than one of
the aforementioned modifications may be incorporated in a
single oligonucleotide or even at within a single nucleoside
within an oligonucleotide. The present invention also
includes oligonucleotides which are chimeric oligonucle-
otides as hereinbefore defined.

The oligonucleotides used in accordance with this inven-
tion may be conveniently and routinely made through the
well-known technique of solid phase synthesis. Equipment
for such synthesis is sold by several vendors including
Applied Biosystems. Any other means for such synthesis
may also be employed; the actual synthesis of the oligo-
nucleotides is well within the talents of the routineer. It is
also well known to use similar techniques to prepare other
oligonucleotides such as the phosphorothioates and alkyl-
ated derivatives. It is also well known to use similar
techniques and commercially available modified amidites
and controlled-pore glass (CPG) products such as biotin,
fluorescein, acridine or psoralen-modified amidites and/or
CPG (available from Glen Research, Sterling Va.) to syn-
thesize fluorescently labeled, biotinylated or other modified
oligonucleotides such as cholesterol-modified oligonucle-
otides.

The oligonucleotides in accordance with this invention
preferably comprise from about 8 to about 50 nucleic acid
base units. In the context of this invention it is understood
that this encompasses non-naturally occurring oligomers as
hereinbefore described, having 8 to 50 monomers.

The oligonucleotides of this invention can be used in
diagnostics, therapeutics and as research reagents and kits.
Since the oligonucleotides of this invention hybridize to the
ras gene, sandwich and other assays can easily be con-
structed to exploit this fact. Furthermore, since the oligo-
nucleotides of this invention hybridize preferentially to the
mutant (activated) form of the ras oncogene, such assays can
be devised for screening of cells and tissues for ras conver-
sion from wild-type to activated form. Such assays can be
utilized for differential diagnosis of morphologically similar
tumors, and for detection of increased risk of cancer stem-
ming from ras gene activation. Provision of means for
detecting hybridization of oligonucleotide with the ras gene
can routinely be accomplished. Such provision may include
enzyme conjugation, radiolabelling or any other suitable
detection systems. Kits for detecting the presence or absence
of nucleic acids encoding ras or activated ras may also be
prepared.

The following specific descriptions serve to illustrate the
invention and are not intended to limit the scope of the
invention:

Antisense Oligonucleotide Inhibition of ras-Luciferase Gene Expression

A series of antisense phosphorothioate oligonucleotides
targeted to either the H-ras translation initiation codon or the
codon-12 point mutation of activated H-ras were screened
using the ras-luciferase reporter gene system described in
Examples 2-5. Of this initial series, six oligonucleotides
were identified that gave significant and reproducible inhi-
bition of ras-luciferase activity. The base sequences,
sequence reference numbers and SEQ ID numbers of these
oligonucleotides (all are phosphorothioates) are shown in
Table 1.

TABLE 1

OLIGO REF NO	SEQUENCE	SEQ ID NO:
2502	CTT-ATA-TTC-CGT-CAT-CGC-TC	1
2503	TCC-GTC-ATC-GCT-CCT-CAG-GG	2
2570	CCA-CAC-CGA-CGG-CGC-CC	3
2571	CCC-ACA-CCG-ACG-GCG-CCC-A	4
2566	GCC-CAC-ACC-GAC-GGC-GCC-CAC	5
2560	TGC-CCA-CAC-CGA-CGG-CGC-CCA-CC	6

A dose-response experiment was performed in which cells
expressing either the normal ras-luciferase reporter gene or
the mutant ras-luciferase reporter gene were treated with
increasing concentrations of the phosphorothioate oligo-
nucleotide 2503 (SEQ ID NO: 2). This compound is targeted
to the translational initiation codon of H-ras RNA tran-
scripts. Treatment of cells with this oligonucleotide resulted
in a dose-dependent inhibition of ras-luciferase activity,
displaying IC50 values of approximately 50 nM for both the
normal and the mutant ras targets. The observation that an
oligonucleotide targeted to the ras translation initiation
codon is equally effective in reducing both mutant and
normal ras expression is expected since the two targets have
identical sequence compositions in the region surrounding
the AUG translation initiation site.

Another dose-response experiment was performed in
which cells were treated with phosphorothioate oligonucle-
otide 2570 (SEQ ID NO: 3), a compound that is targeted to
the codon-12 point mutation of mutant (activated) H-ras
RNA. Treatment of cells with increasing concentrations of
this oligonucleotide resulted in a dose-dependent inhibition
of ras-luciferase activity in cells expressing either the mutant
form or the normal form of ras-luciferase. However, oligo-
nucleotide 2570 displayed approximately threefold selectiv-
ity toward the mutant form of ras-luciferase as compared to
the normal form. In fact, 2570 displayed an IC50 value for
the mutant form of ras-luciferase of approximately 100 nM
whereas the same compound displayed an IC50 value of
approximately 250 nM for the unmutated form.

Cells expressing either the normal form or the mutant
form of ras-luciferase were treated with a single dose (0.5
 μ M) of oligonucleotide targeted to either the translation
initiation codon of H-ras or the codon-12 point mutation.
The antisense phosphorothioate oligonucleotides tested are
shown in Table 1. Compound 2503 (SEQ ID NO: 2),
targeted to the ras translational initiation codon, was most
effective in inhibiting ras-luciferase activity, giving approxi-

mately 80% inhibition of both normal and mutant targets. ISIS 2502 gave 30–35% inhibition of both targets. Of the three compounds targeted to the codon-12 point mutation of activated H-ras, only the 17-mer oligonucleotide 2570 (SEQ ID NO: 3) displayed selectivity toward the mutated form of ras-luciferase as compared to the normal form, giving approximately 22% inhibition of the normal target and 68% inhibition of the mutant target. ISIS 2571 gave approximately 60% inhibition of both targets and ISIS 2566 gave 65–70% inhibition of both targets. Table 2 summarizes data obtained with all 13 antisense oligonucleotides targeted to H-ras. A scrambled control oligonucleotide gave no inhibition of either mutant or normal ras and a control oligonucleotide (ISIS 2907; SEQ ID NO: 19) complementary to the codon-12 region of normal ras gave 70% inhibition of the normal target but had no effect on the mutant ras. Shown for each oligonucleotide is its sequence, region to which it is complementary, and its activity in suppressing expression of the ras-luciferase fusion protein (given as IC₅₀, the concentration in nM necessary to give 50% inhibition of ras-luciferase expression). The longer phosphorothioates targeted to the codon-12 point mutation, while displaying substantial antisense activity toward ras-luciferase expression, did not demonstrate selective inhibition of expression of the mutant form of ras-luciferase. Phosphorothioate oligonucleotides targeted to the codon-12 point mutation that were less than 17 nucleotides in length did not show activity to either form of ras-luciferase. These results demonstrate effective antisense activity of phosphorothioate oligonucleotides targeted to ras sequences.

Antisense Oligonucleotides Specifically Hybridizable With the H-ras AUG

Three 20-base phosphorothioate oligonucleotides, targeted to the H-ras AUG codon, were compared for their ability to inhibit ras-luciferase expression in transient transfection assays as described in Examples 2–5. These oligonucleotides, ISIS 2502 (SEQ ID NO: 1), 2503 (SEQ ID NO: 2) and 6186 (SEQ ID NO: 7) shown in Table 2, were tested for inhibition of ras-luciferase expression at a single dose (100 nM) in HeLa cells. All three AUG-targeted oligonucleotides were effective in inhibiting ras-luciferase expression. These three phosphorothioate oligonucleotides were also prepared with a 2'-O-methyl modification on each sugar. The 2'-O-methylated version of ISIS 2503 (SEQ ID NO: 2) also inhibited ras-luciferase expression with an IC₅₀ between 200 and 500 nM. SEQ ID NO: 7 as a 2'-O-methyl gave approximately 40% inhibition at the highest dose (500 nM).

TABLE 2

Antisense oligonucleotides targeted to mutant H-ras
(Oligonucleotide sequences shown 5' to 3')

ISIS #	TARGET	SEQUENCE	IC ₅₀ (nM)	SEQ. ID NO.
2502	AUG	CTTATATTCGTCATCGCTC	750	1
2503	AUG	TCCGTCATCGCTCCTCAGGG	50	2
6186	AUG	TATTCGTCATCGCTCCTCA	—	7
2563	CODON 12	CGACG	—	8
2564	CODON 12	CCGACGG	—	9
2565	CODON 12	ACCGACGGC	—	10

TABLE 2-continued

Antisense oligonucleotides targeted to mutant H-ras
(Oligonucleotide sequences shown 5' to 3')

ISIS #	TARGET	SEQUENCE	IC ₅₀ (nM)	SEQ. ID NO.
2567	CODON 12	CACCGACGGCG	—	11
2568	CODON 12	ACACCGACGGCGC	—	12
2569	CODON 12	CACACCGACGGCGCC	—	13
3426	CODON 12	CCACACCGACGGCGCC	—	14
3427	CODON 12	CACACCGACGGCGCCC	—	15
2570	CODON 12	CCACACCGACGGCGCCC	100	3
3428	CODON 12	CCCACACCGACGGCGCCC	—	16
3429	CODON 12	CCACACCGACGGCGCCCA	—	17
2571	CODON 12	CCCACACCGACGGCGCCCA	250	4
2566	CODON 12	GCCACACCGACGGCGCCAC	250	5
2560	CODON 12	TGCCACACCGACGGCGCCACC	750	6
2561	CODON 12	TTGCCACACCGACGGCGCCACCA	1000	18
2907	CODON 12 (normal)	CCACACCGCCGGCGCCC	—	19

Oligonucleotide Length Affects Antisense Activity and Specificity

Oligonucleotides targeted to the H-ras codon-12 point mutation also were effective in inhibiting expression of ras-luciferase. A series of eleven phosphorothioate oligonucleotides, ranging in length between 5 and 25 bases, were made and tested for ability to inhibit mutant and wild type ras-luciferase in transient transfection assays as described in Examples 2–5. The oligonucleotides are shown in Table 2. At 100 nM oligonucleotide concentration, oligonucleotides 15 bases or greater in length were found to inhibit expression of the mutant H-ras target. Selective inhibition of mutant over wild type ras-luciferase expression was observed for oligonucleotides between 15 and 19 bases in length. The maximum selectivity observed for inhibition of mutant ras-luciferase relative to wild type was for the 17-mer 2570 (SEQ ID NO: 3) and was approximately 4-fold. In order to demonstrate that 2570 was acting in a sequence-specific manner, a variant of this compound was tested (2907; SEQ ID NO: 19) in which the central adenosine residue was replaced with cytosine, making this oligonucleotide perfectly complementary to the normal H-ras target. Hence, this oligonucleotide will contain a single mismatch at the center of the oligonucleotide/RNA duplex when fully hybridized to the mutant H-ras sequence. Oligonucleotide 2907 selectively inhibited expression of normal ras-luciferase (88% inhibition) relative to mutant ras-luciferase (5% inhibition).

Two 16-mers and two 18-mers complementary to the mutant codon-12 region (Table 2) were tested as described in Examples 2–5. FIG. 1 shows the results of an experiment in which antisense activity and mutant selectivity was determined for oligonucleotides of length 13, 15, 16, 17, 18 and 19 bases in a dose-dependent manner. The results obtained with these oligonucleotides demonstrated that the compounds that were active against mutant H-ras sequences also showed selectivity; oligonucleotides of length 16 (SEQ ID

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NO: 14 and SEQ ID NO: 15) and 17 bases (SEQ ID NO: 3) displayed the greatest selectivity (4- and 5-fold, respectively). The 13 base compound, 2568 (SEQ ID NO: 12), did not display antisense activity at any of the concentrations.

Chimeric 2'-O-methyl Oligonucleotides With Deoxy Gaps

Based on the sequence of the mutant-selective 17-mer (2570), a series of chimeric phosphorothioate 2'-O-methyl oligonucleotides were synthesized in which the end regions consisted of 2'-O-methyl nucleosides and the central residues formed a "deoxy gap". The number of deoxy residues ranged from zero (full 2'-O-methyl) to 17 (full deoxy). These oligonucleotides are shown in Table 3.

TABLE 3

Chimeric phosphorothioate oligonucleotides having 2'-O-methyl ends (bold) and central deoxy gap (Mutant codon-12 target)			
OLIGO #	DEOXY	SEQUENCE	SEQ ID NO
4122	0	CCACACCGACGGCGCCC	3
3975	1	CCACACCGACGGCGCCC	3
3979	3	CCACACCGACGGCGCCC	3
4236	4	CCACACCGACGGCGCCC	3
4242	4	CCACACCGACGGCGCCC	3
3980	5	CCACACCGACGGCGCCC	3
3985	7	CCACACCGACGGCGCCC	3
3984	9	CCACACCGACGGCGCCC	3
2570	17	CCACACCGACGGCGCCC	3

These oligonucleotides were characterized for hybridization efficiency as described in Example 6, ability to direct RNase H cleavage in vitro using mammalian RNase H as described in Example 8, and for antisense activity. Antisense activity against full length H-ras mRNA was determined using a transient co-transfection reporter gene system in which H-ras gene expression was monitored using a ras-responsive enhancer element linked to the reporter gene luciferase, as described in Example 9.

Antisense Activity of Deoxy-gapped Oligonucleotides Against Full Length ras mRNA

The beneficial properties of enhanced target affinity conferred by 2'-O-methyl modifications can be exploited for antisense inhibition provided these compounds are equipped with RNase H-sensitive deoxy gaps of the appropriate length. 2'-O-methyl deoxy gap oligonucleotides were tested for antisense activity against the full length H-ras mRNA using the H-ras transactivation reporter gene system described in Example 9. Antisense experiments were performed initially at a single oligonucleotide concentration (100 nM). Chimeric 2'-O-methyl oligonucleotides containing deoxy gaps of five or more residues inhibited H-ras gene expression. The full deoxy compound gave approximately 50% inhibition. The fully 2'-O-methyl, 1-deoxy and 3-deoxy gave no inhibition. The 5-deoxy, 7-deoxy and 9-deoxy compounds gave approximately 85%, 95% and 90% inhibition, respectively. These compounds displayed activities greater than that of the full deoxy parent compound.

Dose response experiments were performed using these active compounds, along with the 2'-O-methyl chimeras containing four deoxy residues. Oligonucleotide-mediated inhibition of full-length H-ras by these oligonucleotides was

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dose-dependent. The most active compound was the seven-residue deoxy chimera, which displayed an activity approximately five times greater than that of the full deoxy oligonucleotide.

Shortened Chimeric Oligonucleotides

Enhanced target affinity conferred by the 2'-O-methyl modifications was found to confer activity on short chimeric oligonucleotides. A series of short 2'-O-methyl chimeric oligonucleotides were tested for T_m and antisense activity vs. full length ras as described in Example 9. Table 4 shows T_m s for oligonucleotides 11, 13, 15 and 17 nucleotides in length, having deoxy gaps either 5 bases long or 7 bases long. In sharp contrast to the full deoxy 13-mer, both 2'-O-methyl chimeric 13-mers inhibited ras expression, and one of the 11-mers was also active. This is shown in FIG. 2.

TABLE 4

LENGTH	T_m ($^{\circ}$ C.)	SEQUENCE	SEQ ID NO:
17	77.2	CCACACCGACGGCGCCC	3
15	69.8	CACACCGACGGCGCC	13
13	62.1	ACACCGACGGCGC	12
11	47.3	CACCGACGGCG	11
17	74.6	CCACACCGACGGCGCCC	3
15	66.2	CACACCGACGGCGCC	13
13	58.0	ACACCGACGGCGC	12
11	27.7	CACCGACGGCG	11

Relative antisense activity and ability to activate RNase H cleavage in vitro by chimeric 2'-O-methyl oligonucleotides is well correlated with deoxy length (FIG. 3).

Asymmetrical Deoxy Gaps

It is not necessary that the deoxy gap be in the center of the chimeric molecule. It was found that chimeric molecules having the nucleotides of the region at one end modified at the 2' position to enhance binding and the remainder of the molecule unmodified (2'deoxy) can still inhibit ras expression. Oligonucleotides of SEQ ID NO: 3 (17-mer complementary to mutant codon 12) in which a 7-deoxy gap was located at either the 5' or 3' side of the 17-mer, or at different sites within the middle of the molecule, all demonstrated RNase H activation and antisense activity. However, a 5-base gap was found to be more sensitive to placement, as some gap positions rendered the duplex a poor activator of RNase H and a poor antisense inhibitor. Therefore, a 7-base deoxy gap is preferred.

Other Sugar Modifications

The effects of other 2' sugar modifications besides 2'-O-methyl on antisense activity in chimeric oligonucleotides have been examined. These modifications are listed in Table 5, along with the T_m values obtained when 17-mer oligonucleotides having 2'-modified nucleotides flanking a 7-base deoxy gap were hybridized with a 25-mer oligoribonucleotide complement as described in Example 6. A relationship was observed for these oligonucleotides between alkyl length at the 2' position and T_m . As alkyl length increased, T_m decreased. The 2'-fluoro chimeric oligonucleotide displayed the highest T_m of the series.

TABLE 5

Correlation of T _m with Antisense Activity 2'-modified 17-mer with 7-deoxy gap CCACACCGACGGCGCCC (SEQ ID NO: 3)		
2' MODIFICATION	T _m (° C.)	IC50 (nM)
Deoxy	64.2	150
O-Pentyl	68.5	150
O-Propyl	70.4	70
O-Methyl	74.7	20
Fluoro	76.9	10

These 2' modified oligonucleotides were tested for antisense activity against H-ras using the transactivation reporter gene assay described in Example 9. As shown in Table 5, all of these 2' modified chimeric compounds inhibited ras expression, with the 2'-fluoro 7-deoxy-gap compound the most active. A 2'-fluoro chimeric oligonucleotide with a centered 5-deoxy gap was also active.

Chimeric phosphorothioate oligonucleotides having SEQ ID NO: 3 having 2'-O-propyl regions surrounding a 5-base or 7-base deoxy gap were compared to 2'-O-methyl chimeric oligonucleotides. ras expression in T24 cells was inhibited by both 2'-O-methyl and 2'-O-propyl chimeric oligonucleotides with a 7-deoxy gap and a uniform phosphorothioate backbone. When the deoxy gap was decreased to five nucleotides, only the 2'-O-methyl oligonucleotide inhibited ras expression.

Antisense Oligonucleotide Inhibition of H-ras Gene Expression in Cancer Cells

Two phosphorothioate oligonucleotides (2502, 2503) complementary to the ras AUG region were tested as described in Example 10, along with chimeric oligonucleotides (4998, 5122) having the same sequence and 7-base deoxy gaps flanked by 2'-O-methyl regions. These chimeric oligonucleotides are shown in Table 6.

TABLE 6

Chimeric phosphorothioate oligonucleotides having 2'-O-methyl ends (bold) and central deoxy gap (AUG target)			
OLIGO #	DEOXY	SEQUENCE	SEQ ID NO:
2502	20	CTTATATTCGTCATCGCTC	1
4998	7	CTTATATTCGTCATCGCTC	1
2503	20	TCCGTCATCGCTCCTCAGGG	2
5122	7	TCCGTCATCGCTCCTCAGGG	2

Compound 2503 inhibited ras expression in T24 cells by 71%, and the chimeric compound (4998) inhibited ras mRNA even further (84% inhibition). Compound 2502, also complementary to the AUG region, decreased ras RNA levels by 26% and the chimeric version of this oligonucleotide (5122) demonstrated 15% inhibition. Also included in this assay were two oligonucleotides targeted to the mutant codon 12. Compound 2570 (SEQ ID NO: 3) decreased ras RNA by 82% and the 2'-O-methyl chimeric version of this oligonucleotide with a seven-deoxy gap (3985) decreased ras RNA by 95%.

Oligonucleotides 2570 and 2503 were also tested to determine their effects on ras expression in HeLa cells,

which have a wild-type (i.e., not activated) H-ras codon 12. While both of these oligonucleotides inhibited ras expression in T24 cells (having activated codon 12), only the oligonucleotide (2503) specifically hybridizable with the ras AUG inhibited ras expression in HeLa cells. Oligonucleotide 2570 (SEQ ID NO: 3), specifically hybridizable with the activated codon 12, did not inhibit ras expression in HeLa cells, because these cells lack the activated codon-12 target.

Oligonucleotide 2570, a 17-mer phosphorothioate oligonucleotide complementary to the codon 12 region of activated H-ras, was tested for inhibition of ras expression (as described in Example 10) in T24 cells along with chimeric phosphorothioate 2'-O-methyl oligonucleotides 3980, 3985 and 3984, which have the same sequence as 2570 and have deoxy gaps of 5, 7 and 9 bases, respectively (shown in Table 3). The fully 2'-deoxy oligonucleotide 2570 and the three chimeric oligonucleotides decreased ras mRNA levels in T24 cells. Compounds 3985 (7-deoxy gap) and 3984 (9-deoxy gap) decreased ras mRNA by 81%; compound 3980 (5-deoxy gap) decreased ras mRNA by 61%. Chimeric oligonucleotides having this sequence, but having 2'-fluoro-modified nucleotides flanking a 5-deoxy (4689) or 7-deoxy (4690) gap, inhibited ras mRNA expression in T24 cells, with the 7-deoxy gap being preferred (82% inhibition, vs 63% inhibition for the 2'-fluoro chimera with a 5-deoxy gap).

Antisense Oligonucleotide Inhibition of Proliferation of Cancer Cells

Three 17-mer oligonucleotides having the same sequence (SEQ ID NO: 3), complementary to the codon 12 region of activated ras, were tested for effects on T24 cancer cell proliferation as described in Example 11. 3985 has a 7-deoxy gap flanked by 2'-O-methyl nucleotides, and 4690 has a 7-deoxy gap flanked by 2'-F nucleotides (all are phosphorothioates). Effects of these oligonucleotides on cancer cell proliferation correlated well with their effects on ras mRNA expression shown by Northern blot analysis: oligonucleotide 2570 inhibited cell proliferation by 61%, the 2'-O-methyl chimeric oligonucleotide 3985 inhibited cell proliferation by 82%, and the 2'-fluoro chimeric analog inhibited cell proliferation by 93%.

In dose-response studies of these oligonucleotides on cell proliferation, the inhibition was shown to be dose-dependent in the 25 nM–100 nM range. IC50 values of 44 nM, 61 nM and 98 nM could be assigned to oligonucleotides 4690, 3985 and 2570, respectively. The random oligonucleotide control had no effect at the doses tested.

The effect of ISIS 2570 on cell proliferation was cell type-specific. The inhibition of T24 cell proliferation by this oligonucleotide was four times as severe as the inhibition of HeLa cells by the same oligonucleotide (100 nM oligonucleotide concentration). ISIS 2570 is targeted to the activated (mutant) ras codon 12, which is present in T24 but lacking in HeLa cells, which have the wild-type codon 12.

Chimeric Backbone-modified Oligonucleotides

Oligonucleotides discussed in previous examples have had uniform phosphorothioate backbones. The 2'-modified chimeric oligonucleotides discussed above are not active in uniform phosphodiester backbones. A chimeric oligonucleotide was synthesized (ISIS 4226) having 2'-O-methyl regions flanking a 5-nucleotide deoxy gap, with the gap region having a P=S backbone and the flanking regions having a P=O backbone. Another chimeric oligonucleotide (ISIS 4223) having a P=O backbone in the gap and P=S in flanking regions was also made. These oligonucleotides are shown in Table 7.

Additional oligonucleotides were synthesized, completely 2'-deoxy and having phosphorothioate backbones containing either a single phosphodiester (ISIS 4248), two phosphodiester (ISIS 4546), three phosphodiester (ISIS 4551), four phosphodiester (ISIS 4593), five phosphodiester (ISIS 4606) or ten phosphodiester linkages (ISIS-4241) in the center of the molecule. These oligonucleotides are also shown in Table 7.

TABLE 7

Chimeric backbone (P=S/P=O) oligonucleotides having 2'-O-methyl ends (bold) and central deoxy gap (backbone linkages indicated by s (P=S) or o (P=O)) Mutant codon-12 target			
OLIGO #	P=S	SEQUENCE	SEQ ID NO:
2570	16	CsCsAsCsAsCsCsGsAsCsGsGsCsGsCsCsC	3
4226	5	CoCoAoCoAoCsCsGsAsCsGoGoCoGoCoCoC	3
4233	11	CsCsAsCsAsCsCsCsGsAoCsGsGsCsGsCsCsC	3
4248	15	CsCsAsCsAsCsCsGsAoCsGsGsCsGsCsCsC	3
4546	14	CsCsAsCsAsCsCsGsAoCsGsGsCsGsCsCsC	3
4551	13	CsCsAsCsAsCsCsGsAoCsGsGsCsGsCsCsC	3
4593	12	CsCsAsCsAsCsCsGsAoCsGsGsCsGsCsCsC	3
4606	11	CsCsAsCsAsCsCsGsAoCsGsGsCsGsCsCsC	3
4241	6	CsCsAsCsAoCoCoGoAoCoGoGoCoGsCsCsC	3

Oligonucleotides were incubated in crude HeLa cellular extracts at 37° C. to determine their sensitivity to nuclease degradation as described in Dignam et al., *Nucleic Acids Res.* 1983, 11, 1475-1489. The oligonucleotide (4233) with a five-diester gap between phosphorothioate/2'-O-methyl wings had a $T_{1/2}$ of 7 hr. The oligonucleotide with a five-phosphorothioate gap in a phosphorothioate/2'-O-methyl molecule had a $T_{1/2}$ of 30 hours. In the set of oligonucleotides having one to ten diester linkages, the oligonucleotide (4248) with a single phosphodiester linkage was as stable to nucleases as was the full-phosphorothioate molecule, ISIS 2570, showing no degradation after 5 hours in HeLa cell extract. Oligonucleotides with two-, three- and four-diester gaps had $T_{1/2}$ of approximately 5.5 hours, 3.75 hours, and 3.2 hours, and oligonucleotides with five or ten deoxy linkages had $T_{1/2}$ of 1.75 hours and 0.9 hours, respectively.

Antisense Activity of Chimeric Backbone-modified Oligonucleotides

A uniform phosphorothioate backbone is not required for antisense activity. ISIS 4226 and ISIS 4233 were tested in the ras-luciferase reporter system for effect on ras expression as described in Examples 2-5, along with ISIS 2570 (fully phosphorothioate/all deoxy), ISIS 3980 (fully phosphorothioate, 2'-O-methyl wings with deoxy gap) and ISIS 3961 (fully phosphodiester, 2'-O-methyl wings with deoxy gap). All of the oligonucleotides having a P=S (i.e., nuclease-resistant) gap region inhibited ras expression. The two completely 2'-deoxy oligonucleotides having phosphorothioate backbones containing either a single phosphodiester (ISIS 4248) or ten phosphodiester linkages (ISIS 4241) in the center of the molecule were also assayed for activity.

The compound containing a single P=O was just as active as a full P=S molecule, while the same compound containing ten P=O was completely inactive.

Chimeric phosphorothioate oligonucleotides of SEQ ID NO: 3 were made, having a phosphorothioate backbone in the 7-base deoxy gap region only, and phosphodiester in the flanking regions, which were either 2'-O-methyl or 2'-O-propyl. The oligonucleotide with the 2'-O-propyl diester flanking regions was able to inhibit ras expression.

Inhibition of ras-luciferase Gene Expression by Antisense Oligonucleotides Containing Modified Bases

A series of antisense phosphorothioate oligonucleotides complementary to the codon-12 point mutation of activated ras were synthesized as described, having a 2-(amino) adenine at the position complementary to the uracil of the mutated codon 12. Because the amino group at the 2-position of the adenine is able to hydrogen bond with the oxygen in the 2-position on the uracil, three hydrogen bonds instead of the usual two are formed. This serves to greatly stabilize the hybridization of the 2-(amino)adenine-modified antisense oligonucleotide to the activated ras gene, while destabilizing or having no net effect on the stability of this oligonucleotide to the wild-type codon 12, because of the modified A-G mismatch at this position. This increases the specificity of the modified oligonucleotide for the desired target.

An oligonucleotide having a single 2,6-(diamino) adenosine at this position in an otherwise unmodified uniform phosphorothioate 17-mer (sequence identical to 2570, SEQ ID NO: 3) was found to be at least as effective an RNase H substrate as the 2570 sequence. It is therefore expected to be an effective antisense molecule. An oligonucleotide having a single 2,6-(diamino)adenosine at this position in a deoxy gapped phosphorothioate oligonucleotide of the same sequence also demonstrates RNase H activation.

In Vivo Anti-tumor Data

ISIS 2503 (SEQ ID NO: 2) has been evaluated for activity against human tumors in vivo as described in Examples 13 and 14. These studies employed a human lung adenocarcinoma cell line (A549) which was subcutaneously implanted into nude mice, resulting in tumor growth at site of implantation. Since these cells do not contain a mutation in the H-ras gene, but do express normal H-ras, only the AUG-directed oligonucleotide ISIS 2503 was evaluated for anti-tumor activity.

In the first study, phosphorothioate oligonucleotides in saline were administered by intraperitoneal injection at a dosage of 20 mg/kg. Drug treatment was initiated at the time tumors first became visible (28 days following tumor cell inoculation) and treatments were performed every other day. As shown in FIG. 4, no effect on tumor growth was observed after treatment with the unrelated control phosphorothioate oligonucleotide ISIS 1082 (SEQ ID NO: 55). However, significant inhibition of tumor growth was observed for the H-ras-specific oligonucleotide ISIS 2503 (SEQ ID NO: 2). The anti-tumor effects of the H-ras compound were first observed 20 days following initiation of drug treatment and continued throughout the duration of the study.

In a second study, phosphorothioate oligonucleotides were prepared in a cationic lipid formulation (DMRIE:DOPE) and administered by subcutaneous injection as described in Example 15. Drug treatment was initiated one week following tumor cell inoculation and was performed three times a week for only four weeks. As was observed in the first study, administration of the H-ras-specific compound ISIS 2503 (SEQ ID NO: 2) caused a marked reduction in tumor growth whereas the unrelated control oligonucleotide (ISIS 1082) had no significant effect (FIG. 5). Reduction in tumor volume was first observed 20 days following appearance of visible tumors and continued over time throughout the remainder of the study.

Stability of 2'-modified Phosphodiester Oligonucleotides in Cells

Modification of oligonucleotides to confer nuclease stability is required for antisense activity in cells. Certain modifications at the 2' position of the sugar have been found to confer nuclease resistance sufficient to elicit antisense effects in cells without any backbone modification. A uniformly 2'-propoxy modified phosphodiester oligonucleotide (SEQ ID NO: 3) was found to inhibit H-ras expression in T24 cells, 24 hours after administration, at a level equivalent to a phosphorothioate 2'-deoxyoligonucleotide having the same sequence. Uniform 2'-methoxy phosphodiester oligonucleotide also showed some activity. 2'-pentoxy modifications were found to be at least as active as the 2'-propoxy. Antisense Oligonucleotides Active Against Ki-ras

Oligonucleotides were designed to be complementary to the 5'-untranslated region, 3'-untranslated region and coding region of the human Ki-ras oncogene. McGrath, J. P. et al. *Nature* 1983, 304, 501-506. Of the latter, oligonucleotides were targeted to codons 12 and 61 which are known sites of mutation that lead to Ki-ras-mediated transformation, and also to codon 38, which is not known to be involved in transformation. The oligonucleotides are shown in Table 8.

TABLE 8

Antisense Oligonucleotides Complementary to Human Ki-ras			
ISIS #	SEQUENCE	TARGET	SEQ ID NO:
6958	CTG CCT CCG CCG CCG CC	5' UTR/5' cap	20
6957	CAG TGC CTG CGC CGC GCT CG	5'-UTR	21
6956	AGG CCT CTC TCC CGC ACC TG	5'-UTR	22
6953	TTC AGT CAT TTT CAG CAG GC	AUG	23
6952	TTA TAT TCA GTC ATT TTC AG	AUG	24

TABLE 8-continued

Antisense Oligonucleotides Complementary to Human Ki-ras			
ISIS #	SEQUENCE	TARGET	SEQ ID NO:
6951	CAA GTT TAT ATT CAG TCA TT	AUG	25
6950	GCC TAC GCC ACC AGC TCC AAC	Codon 12 (WT)	26
6949	CTA CGC CAC CAG CTC CA	Codon 12 (WT)	27
6948	G TAC TCC TCT TGA CCT GCT GT	Codon 61 (WT)	28
6947	CCT GTA GGA ATC CTC TAT TGT	Codon 38	29
6946	GGT AAT GCT AAA ACA AAT GC	3'-UTR	30
6945	GGA ATA CTG GCA CTT CGA GG	3'-UTR	31
7453	TAC GCC AAC AGC TCC	Codon 12 (G→T mut.)	32
7679	TTT TCA GCA GGC CTC TCT CC	5'-UTR/AUG	33

Twelve Ki-ras-specific oligonucleotides were screened for antisense activity against three colon carcinoma cell lines that contain a mutation at codon 12 in the Ki-ras oncogene and evaluated by measurement of Ki-ras mRNA levels. As shown in FIG. 6, half of the tested compounds displayed significant activity (at least 40% inhibition) against the Ki-ras transcript, with the most active compounds being targeted to the 5'- and 3'-untranslated regions. However, significant inhibition of Ki-ras expression was also observed for compounds directed against wild type codons 12 and 61. Compounds that displayed significant activity were effective against all three carcinoma cell lines tested.

Dose response analysis of these compounds demonstrated that ISIS 6958 and ISIS 6957, both of which target the 5'-UTR, are the most potent inhibitors of Ki-ras in this series of oligonucleotides. These oligonucleotides were examined for their ability to inhibit proliferation of Ki-ras transformed cell lines. The colon carcinoma cell line SW480 was treated with a single dose of oligonucleotide (200 nM) and cell number was determined over a five-day period. As shown in FIG. 7 both Ki-ras specific oligonucleotides were effective inhibitors of proliferation of SW480 cells, with ISIS 6957 (SEQ ID NO: 21) showing greater activity than ISIS 6958 (SEQ ID NO: 20). This difference in activity correlates well with the inhibition of Ki-ras mRNA expression (FIG. 6). Selectivity of Inhibition of Mutant Ki-ras Relative to Normal Ki-ras

Oligonucleotides targeted to Ki-ras have been examined for their ability to selectively inhibit mutant Ki-ras relative to normal Ki-ras. Two cell lines were employed: the SW480 cell line that expresses mutant Ki-ras (codon 12, G to T transversion) and a cell line (HeLa) that expresses normal Ki-ras. Two oligonucleotides were tested: ISIS 6957, SEQ ID NO: 21, a 20 mer phosphorothioate targeted to the 5'-untranslated region of Ki-ras, and ISIS 7453, SEQ ID NO: 32, a 15 mer phosphorothioate targeted to the Ki-ras codon 12 region. Ki-ras mRNA levels were measured 24 hours after treatment. The codon 12-directed compound was effective in the cell line expressing mutant Ki-ras (87% inhibition vs. 18% inhibition in HeLa cells). However, the Ki-ras oligonucleotide targeted to the 5'-untranslated region was a potent inhibitor (95% inhibition) of Ki-ras expression in

both cell lines. Selectivity for mutant Ki-ras was found to be dependent on oligonucleotide concentration and affinity for the RNA target.

Ki-ras Oligonucleotides With Deoxy Gaps

Phosphorothioate oligonucleotides (SEQ ID NO: 21, targeted to the 5'-untranslated region of Ki-ras) were synthesized with 2'-O-methyl modifications flanking central 2'-deoxy gap regions of 6 or 8 nucleotides in length. Both gapped oligonucleotides were active against Ki-ras expression as determined by Northern blot analysis. A uniformly 2'-O-methylated compound (no deoxy gap) was inactive. An additional oligonucleotide, ISIS 7679 (SEQ ID NO: 33, complementary to the 5' untranslated/AUG region of Ki-ras), was also found to be active when synthesized with a 6- or 8-nucleotide deoxy gap.

2'-Methoxyethoxy Analogs of ISIS 2503 (H-ras)

A series of chimeric oligonucleotides were synthesized with the ISIS 2503 sequence (SEQ ID NO: 2) and various arrangements of 2'-methoxyethoxy (2'-MOE) modifications. These are shown in Table 9. All backbone linkages are phosphorothioates.

TABLE 9

2'-MOE analogs of ISIS 2503 Positions with 2'-MOE are shown in bold		
ISIS #	Sequence (5'--3')	SEQ ID NO:
13905	TCCGTCATCGCTCCTCAGGG	2
13907	TCCGTCATCGCTCCTCAGGG	2
13909	TCCGTCATCGCTCCTCAGGG	2
13911	TCCGTCATCGCTCCTCAGGG	2
13917	TCCGTCATCGCTCCTCAGGG	2
13919	TCCGTCATCGCTCCTCAGGG	2
13920	TCCGTCATCGCTCCTCAGGG	2
13923	TCCGTCATCGCTCCTCAGGG	2
13926	TCCGTCATCGCTCCTCAGGG	2
13927	TCCGTCATCGCTCCTCAGGG	2

These oligonucleotides (except for 13919 and 13927 which have not yet been tested) were tested for the ability to reduce H-ras mRNA levels in T24 cells as described in Example 10 except that oligonucleotide and lipofectin were mixed in OptiMEM and kept at a constant ratio of 2.5 ug/ml lipofectin per 100 nM oligonucleotide. All of the tested compounds had activity comparable to ISIS 2503, the parent compound, with IC₅₀'s of 50 nM or below. For this reason oligonucleotides containing one or more 2'-MOE modifications are preferred for reducing ras expression. Dose responses for these compounds are shown in FIG. 8. ISIS 13177 (TCAGTAATAGCCCCACATGG; SEQ ID NO: 34) is a phosphorothioate oligodeoxynucleotide scrambled control for SEQ ID NO: 2.

MMI Analogs of ISIS 2503 (H-ras)

A series of chimeric oligonucleotides were synthesized with the ISIS 2503 sequence (SEQ ID NO: 2) and various placements of methylene(methylimino)backbone linkages. These are shown in Table 10. For ease of synthesis, dimers incorporating an MMI linkage were used in making these oligonucleotides. Dimers containing MMI backbone linkages are indicated by bold lettering. "o" indicates a phosphodiester linkage between MMI dimers. "," indicates a

phosphorothioate linkage between MMI dimers. All unmarked linkages are phosphorothioates.

TABLE 10

MMI analogs of ISIS 2503		
ISIS #	Sequence (5'--3')	SEQ ID NO:
14896	TCCGTCATCGCTCCTCAGGG	2
14897	TC _o CGTCATCGCTCCTCAG _o GG	2
14898	TC _o CGTCATCGCTCCTCAG _o GG	2
14899	TC _o CG _o TCATCGCTCCTC _o A _o GGG	2
14900	TC _o CG _o TCATCGCTCCTC _o AG _o AG	2

These compounds were tested for their ability to reduce H-ras mRNA levels in T24 cells as described in Example 10 except that oligonucleotide and lipofectin were mixed in OptiMEM and kept at a constant ratio of 2.5 ug/ml lipofectin per 100 nM oligonucleotide. As shown in FIG. 9, all of these compounds were able to reduce mRNA levels by 80% or more at doses of 500 nM and below. ISIS 13177 (SEQ ID NO: 34) is a phosphorothioate oligodeoxynucleotide scrambled control for SEQ ID NO: 2. With the exception of ISIS 14899, all the MMI compounds were more active than the parent deoxyphosphorothioate compound, ISIS 2503. Several compounds (ISIS 14896, 14897, 14898) achieved nearly complete ablation of ras mRNA. Oligonucleotides containing one or more MMI modifications are therefore highly preferred for reducing ras expression.

Antisense Oligonucleotides Active Against N-ras

A series of phosphorothioate oligodeoxynucleotides were designed to target human N-ras using the published sequence (Genbank accession number HSNRARS, x02751). These compounds were tested for their ability to reduce N-ras levels in T24 cells as described in Example 10 except that the probe was an N-ras cDNA probe (purchased from Oncogene Science, Cambridge Mass.; catalog no. HP129) and oligonucleotide and lipofectin were mixed in OptiMEM and kept at a constant ratio of 2.5 ug/ml lipofectin per 100 nM oligonucleotide.

These oligonucleotides, and the percent reduction in N-ras mRNA demonstrated for each, are shown in Table 11. Oligonucleotides shown in bold (SEQ ID NO: 44, 45, 46, 47, 49 and 52) demonstrated greater than 30% reduction of ras mRNA when screened at a 300 nM dose and are considered active in this assay. These sequences are therefore preferred. Of these oligonucleotides 14686, 14687, 14688, 14691 and 14694 (SEQ ID NO: 44, 45, 46, 49 and 52) showed greater than 50% inhibition. Dose response curves were obtained for oligonucleotides 14677, 14678, 14686, 14687, 14688, 14689, 14690, 14891, 14692, 14693, and 14694. These are shown in FIG. 10. As can be seen from the figure, ISIS 14686 and ISIS 14691 (SEQ ID NO: 44 and 49, respectively) gave nearly complete ablation of N-ras mRNA at a 400 nM dose.

TABLE 11

Oligonucleotides targeted to human N-ras			
ISIS #	Sequence (5'--3')	Target Region	% SEQ ID NO:
14677	CCGGGTCCTAGAACTGCAG	5' UTR	0.0 35
14678	TAAATCAGTAAAGAAACCG	5' UTR	0.0 36
14679	GGACACAGTAACCAAGCGGC	5' UTR	0.0 37
14680	AACAGAAGCTACACCAAGGG	5' UTR	0.0 38
14681	CAGACCCATCCATTCCCGTG	5' UTR	0.0 39
14682	GCCAAGAAATCAGACCCATC	5' UTR	0.0 40
14683	AGGGGGAAGATAAAACCGCC	5' UTR	0.0 41
14684	CGCTTCCATTCTTTCGCCAT	5' UTR	0.0 42
14685	CCGCACCCAGACCCGCCCT	5' UTR	0.0 43
14686	CAGCCCCACCAAGGAGCGG	5' UTR	61.0 44
14687	GTCATTTACACCAGCAAGA	AUG	50.2 45
14688	CAGTCATTTACACCAGCAA	AUG	60.5 46
14689	CTCAGTCATTTACACCAGC	AUG	38.4 47
14690	CGTGGGCTTGTCTTGTATCA	Coding	0.2 48
14691	CCATACAACCTGAGTCCCA	3' UTR	58.3 49
14692	CAGACAGCCAAGTGAGGAGG	3' UTR	0.0 50
14693	CCAGGGCAGAAAATAACAG	3' UTR	0.0 51
14694	TTTGTGCTGTGGAAGAAGCC	3' UTR	50.7 52
14695	GCTATTAAATAACAATGCAC	3' UTR	0.0 53
14696	ACTGATCACAGCTATTAAAT	3' UTR	0.0 54

The following examples illustrate the present invention and are not intended to limit the same.

EXAMPLES

Example 1

Synthesis and Characterization of Oligonucleotides

Unmodified oligodeoxynucleotides are synthesized on an automated DNA synthesizer (Applied Biosystems model 380B) using standard phosphoramidite chemistry with oxidation by iodine. β -cyanoethyl-diisopropyl-phosphoramidites are purchased from Applied Biosystems (Foster City, Calif.). For phosphorothioate oligonucleotides, the standard oxidation bottle was replaced by a 0.2 M solution of ^3H -1,2-benzodithiole-3-one 1,1-dioxide in acetonitrile for the stepwise thiation of the phosphite linkages. The thiation cycle wait step was increased to 68 seconds and was followed by the capping step.

2'-methoxy oligonucleotides were synthesized using 2'-methoxy β -cyanoethyl-diisopropyl-phosphoramidites (Chemgenes, Needham, Mass.) and the standard cycle for unmodified oligonucleotides, except the wait step after pulse delivery of tetrazole and base was increased to 360 seconds. Other 2'-alkoxy oligonucleotides were synthesized by a modification of this method, using appropriate 2'-modified amidites such as those available from Glen Research, Inc., Sterling, Va.

2'-fluoro oligonucleotides were synthesized as described in Kawasaki et al., *J. Med. Chem.* 1993, 36, 831-841.

Briefly, the protected nucleoside N⁶-benzoyl-2'-deoxy-2'-fluoroadenosine was synthesized utilizing commercially available 9- β -D-arabinofuranosyladenine as starting material and by modifying literature procedures whereby the 2'- α -fluoro atom is introduced by a S_N2-displacement of a 2'- β -O-triflyl group. Thus N⁶-benzoyl-9- β -D-arabinofuranosyladenine was selectively protected in moderate yield as the 3', 5'-ditetrahydropyranyl (THP) intermediate. Deprotection of the THP and N⁶-benzoyl groups was accomplished using standard methodologies and standard methods were used to obtain the 5'-dimethoxytrityl-(DMT) and 5'-DMT-3'-phosphoramidite intermediates.

The synthesis of 2'-deoxy-2'-fluoroguanosine was accomplished using tetraisopropylidisiloxanyl (TPDS) protected 9- β -D-arabinofuranosylguanine as starting material, and conversion to the intermediate diisobutylarabinofuranosylguanine. Deprotection of the TPDS group was followed by protection of the hydroxyl group with THP to give diisobutyl di-THP protected arabinofuranosylguanine. Selective O-deacylation and triflation was followed by treatment of the crude product with fluoride, then deprotection of the THP groups. Standard methodologies were used to obtain the 5'-DMT- and 5'-DMT-3'-phosphoramidites.

Synthesis of 2'-deoxy-2'-fluorouridine was accomplished by the modification of a known procedure in which 2,2'-anhydro-1- β -D-arabinofuranosyluracil was treated with 70% hydrogen fluoride-pyridine. Standard procedures were used to obtain the 5'-DMT and 5'-DMT-3'-phosphoramidites.

2'-deoxy-2'-fluorocytidine was synthesized via amination of 2'-deoxy-2'-fluorouridine, followed by selective protection to give N⁴-benzoyl-2'-deoxy-2'-fluorocytidine. Standard procedures were used to obtain the 5'-DMT and 5'-DMT-3', phosphoramidites.

2'-(2-methoxyethyl)-modified amidites are synthesized according to Martin, P., *Helv. Chim. Acta* 1995, 78, 486-504. For ease of synthesis, the last nucleotide was a deoxynucleotide. 2'-O-CH₂CH₂OCH₃cytosines may be 5-methyl cytosines.

Synthesis of 5-Methyl Cytosine Monomers

2,2'-Anhydro[1-(β -D-arabinofuranosyl)-5-methyluridine]

5-Methyluridine (ribosylthymine, commercially available through Yamasa, Choshi, Japan) (72.0 g, 0.279 M), diphenylcarbonate (90.0 g, 0.420 M) and sodium bicarbonate (2.0 g, 0.024 M) were added to DMF (300 mL). The mixture was heated to reflux, with stirring, allowing the evolved carbon dioxide gas to be released in a controlled manner. After 1 hour, the slightly darkened solution was concentrated under reduced pressure. The resulting syrup was poured into diethylether (2.5 L), with stirring. The product formed a gum. The ether was decanted and the residue was dissolved in a minimum amount of methanol (ca. 400 mL). The solution was poured into fresh ether (2.5 L) to yield a stiff gum. The ether was decanted and the gum was dried in a vacuum oven (60° C. at 1 mm Hg for 24 h) to give a solid which was crushed to a light tan powder (57 g, 85% crude yield). The material was used as is for further reactions.

2'-O-Methoxyethyl-5-methyluridine

2,2'-Anhydro-5-methyluridine (195 g, 0.81 M), tris(2-methoxyethyl)borate (231 g, 0.98 M) and 2-methoxyethanol (1.2 L) were added to a 2 L stainless steel pressure vessel and placed in a pre-heated oil bath at 160° C. After heating for 48 hours at 155-160° C., the vessel was opened and the solution evaporated to dryness and triturated with MeOH (200 mL). The residue was suspended in hot acetone (1 L). The insoluble salts were filtered, washed with acetone (150 mL) and the filtrate evaporated. The residue (280 g) was dissolved in CH₃CN (600 mL) and evaporated. A silica gel

column (3 kg) was packed in CH_2Cl_2 /acetone/MeOH (20:5:3) containing 0.5% Et_3NH_3 . The residue was dissolved in CH_2Cl_2 (250 mL) and adsorbed onto silica (150 g) prior to loading onto the column. The product was eluted with the packing solvent to give 160 g (63%) of product.

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine

2'-O-Methoxyethyl-5-methyluridine (160 g, 0.506 M) was co-evaporated with pyridine (250 mL) and the dried residue dissolved in pyridine (1.3 L). A first aliquot of dimethoxytrityl chloride (94.3 g, 0.278 M) was added and the mixture stirred at room temperature for one hour. A second aliquot of dimethoxytrityl chloride (94.3 g, 0.278 M) was added and the reaction stirred for an additional one hour. Methanol (170 mL) was then added to stop the reaction. HPLC showed the presence of approximately 70% product. The solvent was evaporated and triturated with CH_3CN (200 mL). The residue was dissolved in CHCl_3 (1.5 L) and extracted with 2x500 mL of saturated NaHCO_3 and 2x500 mL of saturated NaCl . The organic phase was dried over Na_2SO_4 , filtered and evaporated. 275 g of residue was obtained. The residue was purified on a 3.5 kg silica gel column, packed and eluted with EtOAc/Hexane/Acetone (5:5:1) containing 0.5% Et_3NH_3 . The pure fractions were evaporated to give 164 g of product. Approximately 20 g additional was obtained from the impure fractions to give a total yield of 183 g (57%).

3'-O-Acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine (106 g, 0.167 M), DMF/pyridine (750 mL of a 3:1 mixture prepared from 562 mL of DMF and 188 mL of pyridine) and acetic anhydride (24.38 mL, 0.258 M) were combined and stirred at room temperature for 24 hours. The reaction was monitored by tlc by first quenching the tlc sample with the addition of MeOH. Upon completion of the reaction, as judged by tlc, MeOH (50 mL) was added and the mixture evaporated at 35° C. The residue was dissolved in CHCl_3 (800 mL) and extracted with 2x200 mL of saturated sodium bicarbonate and 2x200 mL of saturated NaCl . The water layers were back extracted with 200 mL of CHCl_3 . The combined organics were dried with sodium sulfate and evaporated to give 122 g of residue (approx. 90% product). The residue was purified on a 3.5 kg silica gel column and eluted using EtOAc/Hexane(4:1). Pure product fractions were evaporated to yield 96 g (84%).

3'-Acetyl-2'-O-methoxyethyl-5'-dimethoxytrityl-5-methyl-4-triazoleuridine

A first solution was prepared by dissolving 3'-O-acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine (96 g, 0.144 M) in CH_3CN (700 mL) and set aside. Triethylamine (189 mL, 1.44 M) was added to a solution of triazole (90 g, 1.3 M) in CH_3CN (1 L), cooled to -5° C. and stirred for 0.5 h using an overhead stirrer. POCl_3 was added dropwise, over a 30 minute period, to the stirred solution maintained at 0-10° C., and the resulting mixture stirred for an additional 2 hours. The first solution was added dropwise, over a 45 minute period, to the later solution. The resulting reaction mixture was stored overnight in a cold room. Salts were filtered from the reaction mixture and the solution was evaporated. The residue was dissolved in EtOAc (1 L) and the insoluble solids were removed by filtration. The filtrate was washed with 1x300 mL of NaHCO_3 and 2x300 mL of saturated NaCl , dried over sodium sulfate and evaporated. The residue was triturated with EtOAc to give the title compound.

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine

A solution of 3'-O-acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-4-triazoleuridine (103 g, 0.141

M) in dioxane (500 mL) and NH_4OH (30 mL) was stirred at room temperature for 2 hours. The dioxane solution was evaporated and the residue azeotroped with MeOH (2x200 mL). The residue was dissolved in MeOH (300 mL) and transferred to a 2 liter stainless steel pressure vessel. MeOH (400 mL) saturated with NH_3 gas was added and the vessel heated to 100° C. for 2 hours (tlc showed complete conversion). The vessel contents were evaporated to dryness and the residue was dissolved in EtOAc (500 mL) and washed once with saturated NaCl (200 mL). The organics were dried over sodium sulfate and the solvent was evaporated to give 85 g (95%) of the title compound.

N⁴-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine (85 g, 0.134 M) was dissolved in DMF (800 mL) and benzoic anhydride (37.2 g, 0.165 M) was added with stirring. After stirring for 3 hours, tlc showed the reaction to be approximately 95% complete. The solvent was evaporated and the residue azeotroped with MeOH (200 mL). The residue was dissolved in CHCl_3 (700 mL) and extracted with saturated NaHCO_3 (2x300 mL) and saturated NaCl (2x300 mL), dried over MgSO_4 and evaporated to give a residue (96 g). The residue was chromatographed on a 1.5 kg silica column using EtOAc/Hexane (1:1) containing 0.5% Et_3NH_3 as the eluting solvent. The pure product fractions were evaporated to give 90 g (90%) of the title compound.

N⁴-Benzoyl-2'-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine-3'-amidite

N⁴-Benzoyl-2'-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine (74 g, 0.10 M) was dissolved in CH_2Cl_2 (1 L). Tetrazole diisopropylamine (7.1 g) and 2-cyanoethoxytetra(isopropyl)phosphite (40.5 mL, 0.123 M) were added with stirring, under a nitrogen atmosphere. The resulting mixture was stirred for 20 hours at room temperature (tlc showed the reaction to be 95% complete). The reaction mixture was extracted with saturated NaHCO_3 (1x300 mL) and saturated NaCl (3x300 mL). The aqueous washes were back-extracted with CH_2Cl_2 (300 mL), and the extracts were combined, dried over MgSO_4 and concentrated. The residue obtained was chromatographed on a 1.5 kg silica column using EtOAc/Hexane (3:1) as the eluting solvent. The pure fractions were combined to give 90.6 g (87%) of the title compound.

5-methyl-2'-deoxycytidine (5-me-C) containing oligonucleotides were synthesized according to published methods (Sanghvi et al. *Nucl. Acids Res.* 1993, 21, 3197-3203) using commercially available phosphoramidites (Glen Research, Sterling Va. or ChemGenes, Needham Mass.).

Oligonucleotides having methylene(methylimino) (MMI) backbones are synthesized according to U.S. Pat. No. 5,378, 825, which is coassigned to the assignee of the present invention and is incorporated herein in its entirety. For ease of synthesis, various nucleoside dimers containing MMI linkages were synthesized and incorporated into oligonucleotides. Other nitrogen-containing backbones are synthesized according to WO 92/20823 which is also coassigned to the assignee of the present invention and incorporated herein in its entirety.

Oligonucleotides having amide backbones are synthesized according to De Mesmaeker et al. *Acc. Chem. Res.* 1995, 28, 366-374. The amide moiety is readily accessible by simple and well-known synthetic methods and is compatible with the conditions required for solid phase synthesis of oligonucleotides.

Oligonucleotides with morpholino backbones are synthesized according to U.S. Pat. No. 5,034,506 (Summerton and Weller).

Peptide-nucleic acid (PNA) oligomers are synthesized according to P. E. Nielsen et al. *Science*. 1991, 254, 1497).

After cleavage from the controlled pore glass column (Applied Biosystems) and deblocking in concentrated ammonium hydroxide at 55° C. for 18 hours, the oligonucleotides are purified by precipitation twice out of 0.5 M NaCl with 2.5 volumes ethanol. Synthesized oligonucleotides were analyzed by polyacrylamide gel electrophoresis on denaturing gels and judged to be at least 85% full length material. The relative amounts of phosphorothioate and phosphodiester linkages obtained in synthesis were periodically checked by ³¹P nuclear magnetic resonance spectroscopy, and for some studies oligonucleotides were purified by HPLC, as described by Chiang et al. *J. Biol. Chem.* 1991, 266:18162-18171. Results obtained with HPLC-purified material were similar to those obtained with non-HPLC purified material.

Example 2

ras-Luciferase Reporter Gene Assembly

The ras-luciferase reporter genes described in this study were assembled using PCR technology. Oligonucleotide primers were synthesized for use as primers for PCR cloning of the 5'-regions of exon 1 of both the mutant (codon 12) and non-mutant (wild-type) human H-ras genes. The plasmids pT24-C3, containing the c-H-ras1 activated oncogene (codon 12, GGC→GTC), and pbc-N1, containing the c-H-ras proto-oncogene, were obtained from the American Type Culture Collection (Bethesda, Md.). The plasmid pT3/T7 luc, containing the 1.9 kb firefly luciferase gene, was obtained from Clontech Laboratories (Palo Alto, Calif.). The oligonucleotide PCR primers were used in standard PCR reactions using mutant and non-mutant H-ras genes as templates. These primers produce a DNA product of 145 base pairs corresponding to sequences -53 to +65 (relative to the translational initiation site) of normal and mutant H-ras, flanked by NheI and HindIII restriction endonuclease sites. The PCR product was gel purified, precipitated, washed and resuspended in water using standard procedures.

PCR primers for the cloning of the *P. pyralis* (firefly) luciferase gene were designed such that the PCR product would code for the full-length luciferase protein with the exception of the amino-terminal methionine residue, which would be replaced with two amino acids, an amino-terminal lysine residue followed by a leucine residue. The oligonucleotide PCR primers used for the cloning of the luciferase gene were used in standard PCR reactions using a commercially available plasmid (pT3/T7-Luc) (Clontech), containing the luciferase reporter gene, as a template. These primers yield a product of approximately 1.9 kb corresponding to the luciferase gene, flanked by unique HindIII and BssHII restriction endonuclease sites. This fragment was gel purified, precipitated, washed and resuspended in water using standard procedures.

To complete the assembly of the ras-luciferase fusion reporter gene, the ras and luciferase PCR products were digested with the appropriate restriction endonucleases and cloned by three-part ligation into an expression vector containing the steroid-inducible mouse mammary tumor virus promoter MMTV using the restriction endonucleases NheI, HindIII and BssHII. The resulting clone results in the insertion of H-ras 5' sequences (-53 to +65) fused in frame with the firefly luciferase gene. The resulting expression vector encodes a ras-luciferase fusion product which is expressed under control of the steroid-inducible MMTV promoter. These plasmid constructions contain sequences encoding amino acids 1-22 of activated (RA2) or normal (RA4) H-ras proteins fused in frame with sequences coding

for firefly luciferase. Translation initiation of the ras-luciferase fusion mRNA is dependent upon the natural H-ras AUG codon. Both mutant and normal H-ras luciferase fusion constructions were confirmed by DNA sequence analysis using standard procedures.

Example 3

Transfection of Cells with Plasmid DNA

Transfections were performed as described by Greenberg, M. E., in *Current Protocols in Molecular Biology*, (F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. A. Smith, J. G. Seidman and K. Strahl, eds.), John Wiley and Sons, NY, with the following modifications. HeLa cells were plated on 60 mm dishes at 5×10⁵ cells/dish. A total of 10 µg or 12 µg of DNA was added to each dish, of which 1 µg was a vector expressing the rat glucocorticoid receptor under control of the constitutive Rous sarcoma virus (RSV) promoter and the remainder was ras-luciferase reporter plasmid. Calcium phosphate-DNA coprecipitates were removed after 16-20 hours by washing with Tris-buffered saline [50 mM Tris-Cl (pH 7.5), 150 mM NaCl] containing 3 mM EGTA. Fresh medium supplemented with 10% fetal bovine serum was then added to the cells. At this time, cells were pre-treated with antisense oligonucleotides prior to activation of reporter gene expression by dexamethasone.

Example 4

Oligonucleotide Treatment of Cells

Following plasmid transfection, cells were washed with phosphate buffered saline prewarmed to 37° C. and Opti-MEM containing 5 µg/mL N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA) was added to each plate (1.0 ml per well). Oligonucleotides were added from 50 µM stocks to each plate and incubated for 4 hours at 37° C. Medium was removed and replaced with DMEM containing 10% fetal bovine serum and the appropriate oligonucleotide at the indicated concentrations and cells were incubated for an additional 2 hours at 37° C. before reporter gene expression was activated by treatment of cells with dexamethasone to a final concentration of 0.2 µM. Cells were harvested and assayed for luciferase activity fifteen hours following dexamethasone stimulation.

Example 5

Luciferase Assays

Luciferase was extracted from cells by lysis with the detergent Triton X-100 as described by Greenberg, M. E., in *Current Protocols in Molecular Biology*, (F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. A. Smith, J. G. Seidman and K. Strahl, eds.), John Wiley and Sons, NY. A Dynatech ML1000 luminometer was used to measure peak luminescence upon addition of luciferin (Sigma) to 625 µM. For each extract, luciferase assays were performed multiple times, using differing amounts of extract to ensure that the data were gathered in the linear range of the assay.

Example 6

Melting Curves

Absorbance vs temperature curves were measured at 260 nm using a Gilford 260 spectrophotometer interfaced to an IBM PC computer and a Gilford Response II spectrophotometer. The buffer contained 100 mM Na⁺, 10 mM phosphate and 0.1 mM EDTA, pH 7. oligonucleotide concentration was 4 µM each strand determined from the absorbance at 85° C. and extinction coefficients calculated according to Puglisi and Tinoco, *Methods in Enzymol.* 1989, 180, 304-325. T_m values, free energies of duplex formation and association constants were obtained from fits of data to a two

state model with linear sloping baselines. Petersheim, M. and Turner, D. H., *Biochemistry* 1983, 22, 256-263. Reported parameters are averages of at least three experiments. For some oligonucleotides, free energies of duplex formation were also obtained from plots of T_m^{-1} vs \log_{10} (concentration). Borer, P. N., Dengler, B., Tinoco, I., Jr., and Uhlenbeck, O. C., *J. Mol. Biol.*, 1974, 86, 843-853.

Example 7

Gel Shift Assay

The structured ras target transcript, a 47-nucleotide hairpin containing the mutated codon 12, was prepared and mapped as described in Lima et al., *Biochemistry* 1991, 31, 12055-12061. Hybridization reactions were prepared in 20 μ l containing 100 mM sodium, 10 mM phosphate, 0.1 mM EDTA, 100 CPM of T7-generated RNA (approximately 10 pM), and antisense oligonucleotide ranging in concentration from 1 pM to 10 μ M. Reactions were incubated 24 hours at 37° C. Following hybridization, loading buffer was added to the reactions and reaction products were resolved on 20% native polyacrylamide gels, prepared using 45 mM tris-borate and 1 mM $MgCl_2$ (TBM). Electrophoresis was carried out at 10° C. and gels were quantitated using a Molecular Dynamics Phosphorimager.

Example 8

RNase H Analysis

RNase H assays were performed using a chemically synthesized 25-base oligoribonucleotide corresponding to bases +23 to +47 of activated (codon 12, G→U) H-ras mRNA. The 5' end-labeled RNA was used at a concentration of 20 nM and incubated with a 10-fold molar excess of antisense oligonucleotide in a reaction containing 20 mM Tris-Cl, pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 10 μ g tRNA and 4 U RNasin in a final volume of 10 μ l. The reaction components were preannealed at 37° C. for 15 minutes then allowed to cool slowly to room temperature. HeLa cell nuclear extracts were used as a source of mammalian RNase H. Reactions were initiated by addition of 2 μ g of nuclear extract (5 μ l) and reactions were allowed to proceed for 10 minutes at 37° C. Reactions were stopped by phenol/chloroform extraction and RNA components were precipitated with ethanol. Equal CPMs were loaded on a 20% polyacrylamide gel containing 7M urea and RNA cleavage products were resolved and visualized by electrophoresis followed by autoradiography. Quantitation of cleavage products was performed using a Molecular Dynamics Densitometer.

Example 9

ras Transactivation Reporter Gene System

The expression plasmid pSV2-oli, containing an activated (codon 12, GGC→GTC) H-ras cDNA insert under control of the constitutive SV40 promoter, was a gift from Dr. Bruno Tocque (Rhone-Poulenc Sante, Vitry, France). This plasmid was used as a template to construct, by PCR, a H-ras expression plasmid under regulation of the steroid-inducible mouse mammary tumor virus (MMTV) promoter. To obtain H-ras coding sequences, the 570 bp coding region of the H-ras gene was amplified by PCR. The PCR primers were designed with unique restriction endonuclease sites in their 5'-regions to facilitate cloning. The PCR product containing the coding region of the H-ras codon 12 mutant oncogene was gel purified, digested, and gel purified once again prior to cloning. This construction was completed by cloning the insert into the expression plasmid pMAMneo (Clontech Laboratories, CA).

The ras-responsive reporter gene pRDO53 was used to detect ras expression. Owen et al., *Proc. Natl. Acad. Sci. U.S.A.* 1990, 87, 3866-3870.

Example 10

Northern Blot Analysis of ras Expression in vivo

The human urinary bladder cancer cell line T24 was obtained from the American Type Culture Collection (Rockville Md.). Cells were grown in McCoy's 5A medium with L-glutamine (Gibco BRL, Gaithersburg Md.), supplemented with 10% heat-inactivated fetal calf serum and 50 U/ml each of penicillin and streptomycin. Cells were seeded on 100 mm plates. When they reached 70% confluency, they were treated with oligonucleotide. Plates were washed with 10 ml prewarmed PBS and 5 ml of OptiMEM reduced-serum medium containing 2.5 μ l DOTMA was added. Oligonucleotide was then added to the desired concentration. After 4 hours of treatment, the medium was replaced with McCoy's medium. Cells were harvested 48 hours after oligonucleotide treatment and RNA was isolated using a standard CsCl purification method. Kingston, R. E., in *Current Protocols in Molecular Biology*, (F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. A. Smith, J. G. Seidman and K. Strahl, eds.), John Wiley and Sons, NY.

The human epithelioid carcinoma cell line HeLa 229 was obtained from the American Type Culture Collection (Bethesda, Md.). HeLa cells were maintained as monolayers on 6-well plates in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 100 U/ml penicillin. Treatment with oligonucleotide and isolation of RNA were essentially as described above for T24 cells.

Northern hybridization: 10 μ g of each RNA was electrophoresed on a 1.2% agarose/formaldehyde gel and transferred overnight to GeneBind 45 nylon membrane (Pharmacia LKB, Piscataway, N.J.) using standard methods. Kingston, R. E., in *Current Protocols in Molecular Biology*, (F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. A. Smith, J. G. Seidman and K. Strahl, eds.), John Wiley and Sons, NY. RNA was UV-crosslinked to the membrane. Double-stranded 32 P-labeled probes were synthesized using the Prime a Gene labeling kit (Promega, Madison Wis.). The ras probe was a Sali-NheI fragment of a cDNA clone of the activated (mutant) H-ras mRNA having a GGC-to-GTC mutation at codon-12. The control probe was G3PDH. Blots were prehybridized for 15 minutes at 68° C. with the QuickHyb hybridization solution (Stratagene, La Jolla, Calif.). The heat-denatured radioactive probe (2.5×10^6 counts/2 ml hybridization solution) mixed with 100 μ l of 10 mg/ml salmon sperm DNA was added and the membrane was hybridized for 1 hour at 68° C. The blots were washed twice for 15 minutes at room temperature in $2 \times$ SSC/0.1% SDS and once for 30 minutes at 60° C. with $0.1 \times$ SSC/0.1% SDS. Blots were autoradiographed and the intensity of signal was quantitated using an ImageQuant PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.). Northern blots were first hybridized with the ras probe, then stripped by boiling for 15 minutes in $0.1 \times$ SSC/0.1% SDS and rehybridized with the control G3PDH probe to check for correct sample loading.

Example 11

Antisense Oligonucleotide Inhibition of Proliferation of Cancer Cells

Cells were cultured and treated with oligonucleotide essentially as described in Example 10. Cells were seeded on 60 mm plates and were treated with oligonucleotide in the presence of DOTMA when they reached 70% confluency.

Time course experiment: On day 1, cells were treated with a single dose of oligonucleotide at a final concentration of 100 nM. The growth medium was changed once on day 3 and cells were counted every day for 5 days, using a counting chamber.

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Dose-response experiment: Various concentrations of oligonucleotide (10, 25, 50, 100 or 250 nM) were added to the cells and cells were harvested and counted 3 days later. Oligonucleotides 2570, 3985 and 4690 were tested for effects on T24 cancer cell proliferation.

Example 12

Synthesis of 2-(Amino)adenine-substituted Oligonucleotides

Oligonucleotides are synthesized as in Example 1, with the following exception: at positions at which a 2-(amino) adenine is desired, the standard phosphoramidite is replaced with a commercially available 2-aminodeoxyadenosine phosphoramidite (Chemgenes).

Example 13

Culture of A549 Cells

A549 cells (obtained from the American Type Culture Collection, Bethesda Md.) were grown to confluence in 6-well plates (Falcon Labware, Lincoln Park, N.J.) in Dulbecco's modified Eagle's medium (DME) containing 1 g glucose/liter and 10% fetal calf serum (FCS, Irvine Scientific, Santa Ana, Calif.).

Example 14

Oligonucleotide Treatment of Human Tumor Cells in Nude Mice—Intraperitoneal Injection

Human lung carcinoma A549 cells were harvested and 5×10^6 cells (200 μ l) were injected subcutaneously into the inner thigh of nude mice. Palpable tumors develop in approximately one month. Phosphorothioate oligonucleotides ISIS 2503 and 1082 (unrelated control) were administered to mice intraperitoneally at a dosage of 20 mg/kg body weight, every other day for approximately ten weeks. Mice were monitored for tumor growth during this time.

Example 15

Oligonucleotide Treatment of Human Tumor Cells in Nude Mice—Subcutaneous Injection With Cationic Lipid

Human lung carcinoma A549 cells were harvested and 5×10^6 cells (200 μ l) were injected subcutaneously into the

32

inner thigh of nude mice. Palpable tumors develop in approximately one month. Phosphorothioate oligonucleotides ISIS 2503 and the unrelated control oligonucleotide 1082 (dosage 5 mg/kg), prepared in a cationic lipid formulation (DMRIE/DOPE, 60 mg/kg) were administered to mice subcutaneously at the tumor site. Drug treatment began one week following tumor cell inoculation and was given twice a week for only four weeks. Mice were monitored for tumor growth for a total of nine weeks.

Example 16

Stability of 2' Modified Oligonucleotides in T24 Cells

T24 bladder cancer cells were grown as described in Example 10. Cells were treated with a single dose (1 μ M) of oligonucleotide and assayed for H-ras mRNA expression by Northern blot analysis 24 hours later. Oligonucleotides tested were analogs of ISIS 2570 (SEQ ID NO: 3), a 17 mer targeted to H-ras codon 12.

Example 17

Activity of Ki-ras Oligonucleotides Against Three Colon Carcinoma Cell Lines

Human colon carcinoma cell lines Calu 1, SW480 and SW620 were obtained from the American Type Culture Collection (ATCC) and cultured and maintained as described for HeLa cells in Example 10. Cells were treated with a single dose of oligonucleotide (200 nM) and Ki-ras mRNA expression was measured by Northern blot analysis 24 hours later. For proliferation studies, cells were treated with a single dose of oligonucleotide (200 nM) at day zero and cell number was monitored over a five-day period.

Example 18

Oligonucleotide Inhibition of Mutant vs. Wild-type Ki-ras
SW480 cells were cultured as in the previous example. HeLa cells were cultured as in Example 10. Cells were treated with a single dose (100 nM) of oligonucleotide and mRNA levels were determined by Northern blot analysis 24 hours later.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(iii) NUMBER OF SEQUENCES: 55

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

CTTATATTCC GTCATCGCTC

20

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

-continued

(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

TCCGTCATCG CTCCTCAGGG

20

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

CCACACCGAC GCGCGCC

17

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 19
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

CCCACACCGA CGCGGCCCA

19

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

GCCCACACCG ACGGCGCCCA C

21

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

TGCCACACC GACGGCGCCC ACC

23

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

-continued

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

TATTCGGTCA TCGCTCCTCA 20

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

CGACG 5

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 7
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

CCGACGG 7

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 9
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

ACCGACGGC 9

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 11
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

CACCGACGGC G 11

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 13
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

-continued

ACACCGACGG CGC

13

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 15
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

CACACCGACG GCGCC

15

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 16
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

CCACACCGAC GCGGCC

16

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 16
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

CACACCGACG GCGCCC

16

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

CCACACCGA CGGCGCCC

18

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

CCACACCGAC GCGCCCA

18

(2) INFORMATION FOR SEQ ID NO: 18:

-continued

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 25
 (B) TYPE: Nucleic Acid
 (C) STRANDEDNESS: Single
 (D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

TTGCCACAC CGACGGCGCC CACCA

25

(2) INFORMATION FOR SEQ ID NO: 19:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 17
 (B) TYPE: Nucleic Acid
 (C) STRANDEDNESS: Single
 (D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

CCACACCGCC GGCGCCC

17

(2) INFORMATION FOR SEQ ID NO: 20:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 20
 (B) TYPE: Nucleic Acid
 (C) STRANDEDNESS: Single
 (D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

CTGCCTCCGC CGCCGCGGCC

20

(2) INFORMATION FOR SEQ ID NO: 21:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 20
 (B) TYPE: Nucleic Acid
 (C) STRANDEDNESS: Single
 (D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

CAGTGCCTGC GCCGCGCTCG

20

(2) INFORMATION FOR SEQ ID NO: 22:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 20
 (B) TYPE: Nucleic Acid
 (C) STRANDEDNESS: Single
 (D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

AGGCCTCTCT CCCGCACCTG

20

(2) INFORMATION FOR SEQ ID NO: 23:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 20
 (B) TYPE: Nucleic Acid

-continued

(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

TTCAGTCATT TTCAGCAGGC

20

(2) INFORMATION FOR SEQ ID NO: 24:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

TTATATTCAG TCATTTTCAG

20

(2) INFORMATION FOR SEQ ID NO: 25:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

CAAGTTTATA TTCAGTCATT

20

(2) INFORMATION FOR SEQ ID NO: 26:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

GCCTACGCCA CCAGCTCCAA C

21

(2) INFORMATION FOR SEQ ID NO: 27:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

CTACGCCACC AGCTCCA

17

(2) INFORMATION FOR SEQ ID NO: 28:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

-continued

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

GTACTCCTCT TGACCTGCTG T

21

(2) INFORMATION FOR SEQ ID NO: 29:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:

CCTGTAGGAA TCCTCTATTG T

21

(2) INFORMATION FOR SEQ ID NO: 30:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30:

GGTAATGCTA AAACAAATGC

20

(2) INFORMATION FOR SEQ ID NO: 31:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 31:

GGAATACTGG CACTTCGAGG

20

(2) INFORMATION FOR SEQ ID NO: 32:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 15
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 32:

TACGCCAACA GCTCC

15

(2) INFORMATION FOR SEQ ID NO: 33:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 33:

TTTTCAGCAG GCCTCTCTCC

20

-continued

(2) INFORMATION FOR SEQ ID NO: 34:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 34:

TCAGTAATAG CCCCATGG

20

(2) INFORMATION FOR SEQ ID NO: 35:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 35:

CCGGGTCCTA GAAGCTGCAG

20

(2) INFORMATION FOR SEQ ID NO: 36:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 36:

TAAATCAGTA AAAGAAACCG

20

(2) INFORMATION FOR SEQ ID NO: 37:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 37:

GGACACAGTA ACCAGGCGGC

20

(2) INFORMATION FOR SEQ ID NO: 38:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 38:

AACAGAAGCT ACACCAAGGG

20

(2) INFORMATION FOR SEQ ID NO: 39:

-continued

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 20
 (B) TYPE: Nucleic Acid
 (C) STRANDEDNESS: Single
 (D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 39:

CAGACCCATC CATTCCCGTG 20

(2) INFORMATION FOR SEQ ID NO: 40:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 20
 (B) TYPE: Nucleic Acid
 (C) STRANDEDNESS: Single
 (D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 40:

GCCAAGAAAT CAGACCCATC 20

(2) INFORMATION FOR SEQ ID NO: 41:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 20
 (B) TYPE: Nucleic Acid
 (C) STRANDEDNESS: Single
 (D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 41:

AGGGGGAAGA TAAACCGCC 20

(2) INFORMATION FOR SEQ ID NO: 42:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 20
 (B) TYPE: Nucleic Acid
 (C) STRANDEDNESS: Single
 (D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 42:

CGCTTCATT CTTTCGCCAT 20

(2) INFORMATION FOR SEQ ID NO: 43:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 20
 (B) TYPE: Nucleic Acid
 (C) STRANDEDNESS: Single
 (D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 43:

CCGCACCCAG ACCCGCCCT 20

(2) INFORMATION FOR SEQ ID NO: 44:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 20
 (B) TYPE: Nucleic Acid
 (C) STRANDEDNESS: Single

-continued

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 44:

CAGCCCCCAC CAAGGAGCGG 20

(2) INFORMATION FOR SEQ ID NO: 45:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 45:

GTCATTTCAC ACCAGCAAGA 20

(2) INFORMATION FOR SEQ ID NO: 46:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 46:

CAGTCATTTT ACACCAGCAA 20

(2) INFORMATION FOR SEQ ID NO: 47:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 47:

CTCAGTCATT TCACACCAGC 20

(2) INFORMATION FOR SEQ ID NO: 48:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 48:

CGTGGGCTTG TTTTGTATCA 20

(2) INFORMATION FOR SEQ ID NO: 49:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

-continued

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 49:

CCATACAACC CTGAGTCCCA

20

(2) INFORMATION FOR SEQ ID NO: 50:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 50:

CAGACAGCCA AGTGAGGAGG

20

(2) INFORMATION FOR SEQ ID NO: 51:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 51:

CCAGGGCAGA AAAATAACAG

20

(2) INFORMATION FOR SEQ ID NO: 52:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 52:

TTTGTGCTGT GGAAGAACCC

20

(2) INFORMATION FOR SEQ ID NO: 53:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 53:

GCTATTAAAT AACAAATGCAC

20

(2) INFORMATION FOR SEQ ID NO: 54:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 54:

ACTGATCACA GCTATTAAAT

20

-continued

(2) INFORMATION FOR SEQ ID NO: 55:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 55:

GCCGAGGTCC ATGTCGTACG C

21

What is claimed is:

1. An oligonucleotide 8 to 30 nucleotides in length which is targeted to a nucleic acid encoding human H-ras and which is capable of inhibiting H-ras expression, wherein said oligonucleotide comprises SEQ ID NO: 1, 2, 3, 4, 5, 7, 11, 12, 13, 14 or 15.

2. The oligonucleotide of claim 1 which comprises at least one backbone modification.

3. The oligonucleotide of claim 1 wherein at least one of the nucleotide units of the oligonucleotide is modified at the 2' position of the sugar.

4. The oligonucleotide of claim 1 which is a chimeric oligonucleotide.

5. The oligonucleotide of claim 1 in a pharmaceutically acceptable carrier.

6. A method of modulating the expression of human H-ras comprising contacting tissues or cells containing a human H-ras gene with an effective amount of an oligonucleotide of claim 1 whereby expression of H-ras is modulated.

7. A method of inhibiting the proliferation of cancer cells comprising contacting cancer cells with an effective amount of an oligonucleotide of claim 1, whereby proliferation of the cancer cells is inhibited.

8. A method of preventing or treating a condition arising from the activation of a ras oncogene comprising contacting an animal suspected of having a condition arising from the activation of a ras oncogene with an effective amount of an oligonucleotide of claim 1, whereby said condition is prevented or treated.

9. An oligonucleotide 8 to 30 nucleotides in length which is targeted to a nucleic acid encoding human Ki-ras and

which is capable of inhibiting Ki-ras expression, wherein said oligonucleotide comprises SEQ ID NO: 20, 21, 22, 26, 28, 31 or 32.

10. The oligonucleotide of claim 9 which comprises at least one backbone modification.

11. The oligonucleotide of claim 9 wherein at least one of the nucleotide units of the oligonucleotide is modified at the 2' position of the sugar.

12. The oligonucleotide of claim 9 which is a chimeric oligonucleotide.

13. The oligonucleotide of claim 9 in a pharmaceutically acceptable carrier.

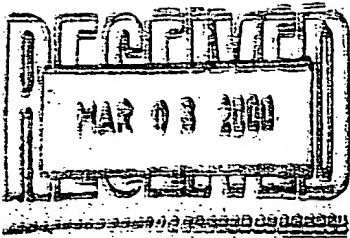
14. A method of modulating the expression of human Ki-ras comprising contacting tissues or cells containing a human Ki-ras gene with an effective amount of an oligonucleotide of claim 9 whereby expression of Ki-ras is modulated.

15. A method of inhibiting the proliferation of cancer cells comprising contacting cancer cells with an effective amount of an oligonucleotide of claim 9, whereby proliferation of the cancer cells is inhibited.

16. A method of preventing or treating a condition arising from the activation of a ras oncogene comprising contacting an animal suspected of having a condition arising from the activation of a ras oncogene with an effective amount of an oligonucleotide of claim 9, whereby said condition is prevented or treated.

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UNITED STATES DEPARTMENT OF COMMERCE
Patent and Trademark Office

NOTICE OF ALLOWANCE AND ISSUE FEE DUE

HM12/0301

JANE MASSEY LICATA
66 E. MAIN STREET
MARLTON NJ 08053

Docket System ☒
Status Report ☒
Docket Book ☒

APPLICATION NO.	FILING DATE	TOTAL CLAIMS	EXAMINER AND GROUP ART UNIT	DATE MAILED
09/128,494	08/03/98	03	FREDMAN, J	03/01/00
First Named Applicant	MONIA,	05 USC 154(b) term ext. =	0 Days.	

TITLE OF INVENTION ANTISENSE OLIGONUCLEOTIDE INHIBITION OF RAS

ATTY'S DOCKET NO.	CLASS-SUBCLASS	BATCH NO.	APPLN. TYPE	SMALL ENTITY	FEE DUE	DATE DUE
1	19PH-0016	614-0442000	197	UTILITY	NO	\$1210.00 08/01/00

THE APPLICATION IDENTIFIED ABOVE HAS BEEN EXAMINED AND IS ALLOWED FOR ISSUANCE AS A PATENT. PROSECUTION ON THE MERITS IS CLOSED.

THE ISSUE FEE MUST BE PAID WITHIN THREE MONTHS FROM THE MAILING DATE OF THIS NOTICE OR THIS APPLICATION SHALL BE REGARDED AS ABANDONED. THIS STATUTORY PERIOD CANNOT BE EXTENDED.

HOW TO RESPOND TO THIS NOTICE:

I. Review the SMALL ENTITY status shown above.

If the SMALL ENTITY is shown as YES, verify your current SMALL ENTITY status:

- A. If the status is changed, pay twice the amount of the FEE DUE shown above and notify the Patent and Trademark Office of the change in status, or
- B. If the status is the same, pay the FEE DUE shown above.

If the SMALL ENTITY is shown as NO:

- A. Pay FEE DUE shown above, or
- B. File verified statement of Small Entity Status before, or with, payment of 1/2 the FEE DUE shown above.

II. Part B-Issue Fee Transmittal should be completed and returned to the Patent and Trademark Office (PTO) with your ISSUE FEE. Even if the ISSUE FEE has already been paid by charge to deposit account, Part B Issue Fee Transmittal should be completed and returned. If you are charging the ISSUE FEE to your deposit account, section "4b" of Part B-Issue Fee Transmittal should be completed and an extra copy of the form should be submitted.

III. All communications regarding this application must give application number and batch number. Please direct all communications prior to issuance to Box ISSUE FEE unless advised to the contrary.

IMPORTANT REMINDER: Utility patents issuing on applications filed on or after Dec. 12, 1980 may require payment of maintenance fees. It is patentee's responsibility to ensure timely payment of maintenance fees when due.

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Notice of Allowability

Application No.

09/128,494

Applicant(s)

Monia et al

Examiner.

Jeffrey Fredman

Group Art Unit

1655



All claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance and Issue Fee Due or other appropriate communication will be mailed in due course.

☒ This communication is responsive to December 27, 1999

☒ The allowed claim(s) is/are 13 and 18-32

☐ The drawings filed on _____ are acceptable.

☐ Acknowledgement is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d).

☐ All ☐ Some* ☐ None of the CERTIFIED copies of the priority documents have been
☐ received.

☐ received in Application No. (Series Code/Serial Number) _____

☐ received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

*Certified copies not received: _____

☐ Acknowledgement is made of a claim for domestic priority under 35 U.S.C. § 119(e).

A SHORTENED STATUTORY PERIOD FOR RESPONSE to comply with the requirements noted below is set to EXPIRE THREE MONTHS FROM THE "DATE MAILED" of this Office action. Failure to timely comply will result in ABANDONMENT of this application. Extensions of time may be obtained under the provisions of 37 CFR 1.136(a).

☐ Note the attached EXAMINER'S AMENDMENT or NOTICE OF INFORMAL APPLICATION, PTO-152, which discloses that the oath or declaration is deficient. A SUBSTITUTE OATH OR DECLARATION IS REQUIRED.

☒ Applicant MUST submit NEW FORMAL DRAWINGS

☒ because the originally filed drawings were declared by applicant to be informal.

☐ including changes required by the Notice of Draftsperson's Patent Drawing Review, PTO-948, attached hereto or to Paper No. _____

☐ including changes required by the proposed drawing correction filed on _____, which has been approved by the examiner.

☐ including changes required by the attached Examiner's Amendment/Comment.

Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the reverse side of the drawings. The drawings should be filed as a separate paper with a transmittal letter addressed to the Official Draftsperson.

☐ Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

Any response to this letter should include, in the upper right hand corner, the APPLICATION NUMBER (SERIES CODE/SERIAL NUMBER). If applicant has received a Notice of Allowance and Issue Fee Due, the ISSUE BATCH NUMBER and DATE of the NOTICE OF ALLOWANCE should also be included.

Attachment(s)

☐ Notice of References Cited, PTO-892

☐ Information Disclosure Statement(s), PTO-1449, Paper No(s). _____

☐ Notice of Draftsperson's Patent Drawing Review, PTO-948

☐ Notice of Informal Patent Application, PTO-152

☒ Interview Summary, PTO-413

☐ Examiner's Amendment/Comment

☐ Examiner's Comment Regarding Requirement for Deposit of Biological Material

☒ Examiner's Statement of Reasons for Allowance

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REASONS FOR ALLOWANCE

1. The following is an examiner's statement of reasons for allowance: The claimed invention is drawn to oligonucleotides with sequences which minimally comprise the complete sequence of SEQ ID NOs: 1-5, 7, 11-15, 20-22, 26, 28, 31 and 32 and methods of using these sequences in antisense therapy methods. Applicant demonstrates that for these specific sequences, the secondary consideration of unexpected results overcomes the prima facie obviousness of the sequences in view of Bos, Daaka, Hall and Saiso-Behmoaras in view of Uhlmann, Agrawal, Inoue and further in view of Smith. The cited prior art teaches synthesis of Ras oligonucleotides to inhibit ras induced cellular proliferation and specifically identifies general sites within the Ras sequence which would be desirable for use in such methods. However, as MPEP 2144.09 states "A prima facie case of obviousness based on structural similarity is rebuttable by proof that the claimed compounds possess unexpectedly advantageous or superior properties. In re Papesch, 315 F.2d 381, 137 USPQ 43 (CCPA 1963)." Here, Figure 6 and Table 2 on page 20 of the specification clearly shows that the claimed compounds functioned to reduce Ras expression in eukaryotic cells while other compounds selected from equivalent regions failed to achieve such a reduction. Further, applicant has demonstrated a correlation for some anti-ras antisense oligonucleotides and inhibition of cellular proliferation and of in vivo tumor growth in animals. For these reasons, the specific oligonucleotides and methods of using them are novel and unobvious over the cited prior art.

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Any comments considered necessary by applicant must be submitted no later than the payment of the issue fee and, to avoid processing delays, should preferably accompany the issue fee. Such submissions should be clearly labeled "Comments on Statement of Reasons for Allowance."

2. Any inquiry concerning this communication or earlier communications from the examiner should be directed to Jeff Fredman, Ph.D. whose telephone number is (703) 308-6568.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, W. Gary Jones, can be reached on (703) 308-1152.

Any inquiry of a general nature or relating to the status of this application should be directed to the Technology Center receptionist whose telephone number is (703) 308-0196.

Papers related to this application may be submitted to Technology Center 1600 by facsimile transmission via the P.T.O. Fax Center located in Crystal Mall 1. The CM1 Fax Center numbers for Technology Center 1600 are either (703) 305-3014 or (703) 308-4242. Please note that the faxing of such papers must conform with the Notice to Comply published in the Official Gazette, 1096 OG 30 (November 15, 1989).



Jeffrey Fredman
Patent Examiner
Art Unit 1655

February 24, 2000

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